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BACILLUS STEAROTHERMOPHILUS IN HERRING STICKWATER[1]

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Abstract

Forty-five cultures of thermophilic aerobic sporeforming bacilli isolated from samples of herring stickwater have been identified as strains of *Bacillus stearothermophilus* Donk.

In a study of the conversion of herring fish stickwater to solubles, MacLeod, Idler, and Thomson (4) noted the occurrence of large numbers of aerobic and anaerobic bacteria capable of growing at 60° C. They observed that in a 48 hour period at this temperature there was a 15 to 20% loss in total solids in the stickwater with an accompanying increase in bacterial numbers. Campbell (2) isolated 45 cultures of aerobic bacteria from samples of the herring stickwater which he had held at 62° C. for 18 and 48 hours. In the present study, these cultures were characterized.

Media and Methods

The media and techniques employed for classification are those used by Gordon and Smith (3) and Smith, Gordon, and Clark (5) with the following exceptions. As suggested by Allen (1), 0.01% yeast extract was added to the glucose–nitrate and the citrate media to provide the growth factors required by some of the thermophilic bacilli. Parallel inoculations into similar media with the nitrate or the citrate omitted served as controls. Starch hydrolysis was determined by flooding the plates with approximately 0.01 normal iodine - potassium iodide solution and the zone of clearing noted. All cultures were incubated at 60° C. and the appropriate tests made at 18 to 36 hours; representative cultures were checked for nitrate reduction and acetylmethylcarbinol production at shorter and longer incubation times but the results did not vary. Temperature limits of growth were determined in a covered constant temperature water bath. Cell measurements were made from preparations of 4 and 8 hour agar slope cultures stained by Gram's method.

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Results

The cultures represent strains of *Bacillus stearothermophilus* and are similar to Allen's Group I isolates. The general characteristics of the cultures were as follows: Vegetative rods, 2–6 × 0.8–1.0μ, Gram positive in 8 hour or younger cultures, spores mostly oval, usually terminal and swelling the sporangium. Temperature limits of growth, 40–41° C. and 70–72° C. Nitrate is not used as a nitrogen source. Starch is hydrolyzed, usually rapidly. Acetylmethylcarbinol and indole are not produced. Gas is not formed from glucose.

Forty of the 45 cultures were further differentiated into five groups, as outlined in Table I. The remaining five cultures each differed from the others in one characteristic.

TABLE I

	Group number				
	1	2	3	4	5
	Number of cultures				
	20	8	5	5	2
Gelatin hydrolyzed	+	+	+	+	+
Casein hydrolyzed	+	+	−	+	+
Acid from glucose	+	+	+	−	+
Final pH in glucose broth	±5.5	±5.5	±5.5	±7.6	±5.5
Nitrate reduced	−	+	−	+	−
Citrate as C source	−	−	−	+	+

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THE ENUMERATION OF THERMOPHILIC BACTERIA BY THE PLATE COUNT METHOD[1]

NORA E. NEILSON, MARY F. MACQUILLAN, AND J. J. R. CAMPBELL

Abstract

The estimation of the numbers of aerobic thermophilic bacteria by the plate count method was found to be greatly influenced by the composition of the diluent and by its temperature. A satisfactory diluent consisted of 0.8% KCl in $M/200$ pH 7.2 potassium phosphate buffer. The need of maintaining the temperature at 37° C. and using the special diluent was demonstrated with pure cultures and with mixed cultures obtained by the enrichment procedure. In some cases, the new condition of plating gave counts 100 times those obtained by conventional means. Cultures which were almost completely free of spores were used in determining the optimum plating conditions.

Introduction

During a study of the physiology of some thermophilic bacteria, it became necessary to determine the numbers of these organisms present in the growth medium. Recommended plating techniques (3) were applied, but inconsistent and low values were obtained. Previous workers have noted the difficulties in estimating the numbers of thermophilic bacteria and have recorded that counts of 10^8 were maximum (1).

The present study was undertaken in an attempt to improve the existing procedure for estimating the numbers of thermophilic bacteria by the plate count method.

Methods

The cultures used have been characterized by Neilson and Christie (2) as being varieties of *Bacillus stearothermophilus*. The turbidimetric readings used for establishing the temperature range of growth were made on test tube cultures of the organism which were not shaken during the incubation period. However, in all other work the broth was dispensed in 10 ml. quantities into 50-ml. Erlenmeyer flasks. These flasks were shaken on a Burrell wrist-action shaker operating at 270 oscillations per minute. In order that turbidimetric readings might be made on the cultures without removal from the flasks, a matched test tube was sealed onto the mouth of each flask. A short side-arm was added to the test tube and this was the only means of access to the flask – test tube combination. The side-arm was stoppered with a cork, thus preventing any detectable evaporation even after 24 hours of shaking at 60° C. The apparatus was cleaned in dichromate cleaning solution. Turbidimetric readings were made in a Fisher Electrophotometer using the 650 filter. When plate counts were to be made, a 0.1 ml. sample was removed from the flask with a 0.1 ml. long tip measuring pipette. The plating medium was Bacto Plate Count Agar with 0.3% Beef Extract added.

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Incubation of the plates was carried out without attention to any particular precautions. No water was placed in the incubator and the plates were not sealed or covered in any special fashion. All platings were carried out in duplicate and repeated on a number of occasions.

Experimental

All of the exploratory work was carried out with organism No. 44 and the general applicability of the results tested with other representative organisms. The optimum growth temperature of organism No. 44 as determined by turbidity measurements at 4 hours in still test tubes was found to be 60° C. (Fig. 1). The same relative picture prevailed when readings were taken at 8 and 12 hours. It was thought that the previous history of the inoculum might influence the results of such an experiment and so subcultures were carried out at both 60° C. and 65° C. for three transfers before the optimum growth temperature was determined. Both variations gave results similar to those recorded in Fig. 1. The six other organisms had almost identical responses to temperature. All further work was carried out with the 50-ml. Erlenmeyer flasks which were shaken in a covered 60° C. water bath. A 0.5% inoculum of a 16 hour culture was introduced into the 10 ml. of medium in the flask. A 4 hour culture grown under these conditions served as the sample in all plating experiments. It is important to note that the cultures employed in this work were almost completely devoid of spores at the time of plating and so interpretation of results must be limited to cultures in the vegetative state.

In an effort to standardize a number of the possible variables in the plating procedure, experiments were set up to determine the most desirable

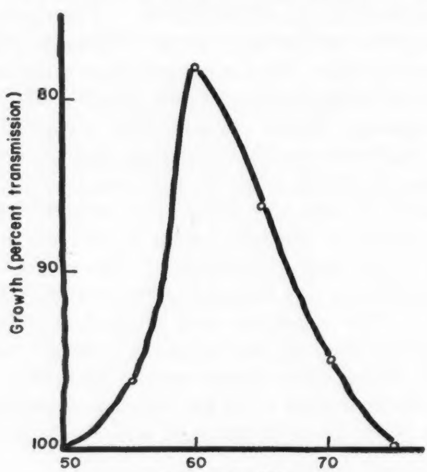

FIG. 1. Optimum growth temperature of *B. stearothermophilus* No. 44.

temperature for incubation of the plates. The final choice was 55° C. Higher temperatures resulted in excessive dehydration of the agar, and lower temperatures required longer incubation periods. Moreover, when mixed populations are used, a selective temperature such as 55° or even higher is essential. With the pure cultures, plates incubated at temperatures ranging from 45° C. to 60° C. gave almost identical counts. The temperature of the agar at time of pouring was varied and 45° C. was chosen as most desirable since higher temperatures resulted in excessive condensation of moisture on the plates. Colonies were counted at 18 hours. Prolonged incubation gave no increase. The amount of agar per plate was varied from 8 to 20 ml. without any influence on the number of colonies appearing. As a convenient amount of agar, 12 ml. was chosen and this amount was dispensed into test tubes in order to keep this factor constant. The composition of the medium, the time and temperature of incubation, and the temperature of the agar at time of pouring were therefore standardized as described.

The use of distilled water as diluent gave low and inconsistent results. The use of $M/200$ pH 7.2 phosphate buffer, 0.8% KCl, or 0.1% gelatin alone and in various combinations greatly improved the values obtained (Table I).

TABLE I

INFLUENCE OF DILUENT ON PLATE COUNT OF *B. stearothermophilus* No. 44*

Time in dilution blank (min.)	Water	Phosphate	KCl	Phosphate–KCl	Gelatin–phosphate	Gelatin–KCl	Gelatin–KCl–phosphate
1	110	640	790	950	970	790	830
10	80	28	360	880	800	730	790

*Counts × 10^6. All diluents were at 37° C.

Neither phosphate nor KCl alone was satisfactory as evidenced by the decreased count on standing. Since there was no great advantage to any one of the other variations, the phosphate–KCl diluent was chosen for further work. Variation of the pH of the buffer, addition of Tween 80, or replacement of potassium by sodium did not improve the diluent.

It should be noted that the dilution blanks were held at 37° C. This is an additional important factor accounting for a twofold increase in the values obtained (Table II).

TABLE II

INFLUENCE OF TEMPERATURE OF DILUENT ON THE PLATE COUNT OF *B. stearothermophilus* No. 44*

Time in dilution blank (min.)	H_2O as diluent			PO_4–KCl as diluent		
	25° C.	37° C.	55° C.	25° C.	37° C.	55° C.
1	104	108	6	360	770	550
15	40	67	0.9	390	630	550

*Counts × 10^6.

TABLE III

COMPARISON OF PLATE COUNT AND TOTAL DIRECT MICROSCOPIC COUNT*

	Plate count†	Direct count‡
Experiment 1	590	600
Experiment 2	810	700

* Counts × 10^6.
†Average of five plates.
‡Average of 30 fields.

When the plate count of the 4 hour culture was carried out using phosphate–KCl diluent at 37° C., the value obtained was similar to that arrived at by the total count direct microscopic method (Table III). These data indicate that at 4 hours there are not many dead cells and that the plate count method determines essentially all of the viable organisms.

To check the general usefulness of our observation, experiments were extended to include six other pure cultures of thermophilic bacteria. The advantages of phosphate–KCl diluent at 37° C. were confirmed (Table IV).

TABLE IV

PHOSPHATE–KCl AS DILUENT FOR ESTIMATING THE NUMBER OF ORGANISMS IN PURE CULTURES OF THERMOPHILES*

	Water		Phosphate–KCl	
Time in diluent	1 min.	30 min.	1 min.	30 min.
Organism No. 4	250	1.5	470	230
Organism No. 70	310	2.0	600	380
Organism No. 83	250	60	750	790
Organism No. 32	1120	530	1130	670
Organism No. 86	280	60	610	620
Organism No. 33	820	140	790	580
Organism No. 44	260	170	450	370

*Counts × 10^6.

In fact, organisms No. 4 and No. 70, which were isolated from raw sugar, were found to be extremely sensitive to distilled water.

Additional data were obtained by plating enrichment cultures obtained from samples of gelatin, manure, greenhouse soil, compost, raw sugar, and sugar refinery sweet water. Approximately 1 gram of each of these source materials was added to 10 ml. of yeast extract – tryptic digest of casein broth in a 125 ml. Erlenmeyer flask and incubated at 60° C. for 18 hours. A transfer was made from these cultures to fresh flasks of media, incubation carried out at 60° C., and samples removed for plating at 4 to 6 hours (Table V). These data indicate that the use of the buffered diluent at 37° C. may give count

TABLE V

INFLUENCE OF DILUENT AND ITS TEMPERATURE ON THE ESTIMATION OF THERMOPHILES IN RAW MATERIALS*

	Temperature of diluent			
	25° C.		37° C.	
	Diluent			
	H_2O	Phosphate–KCl	H_2O	Phosphate–KCl
Source				
Manure (1)	370	680	12,100	40,000
(2)	250	440	190	2,200
Gelatin (1)	72,000	81,000	68,000	115,000
(2)	22,600	80,000	78,000	108,000
Greenhouse soil	480	3,100	2,000	5,500
Compost	4,100	13,600	15,500	105,000
Raw sugar (1)	134,000	290,000	77,000	440,000
(2)	50,000	98,000	24,000	200,000
Sugar refinery sweet water	8,500	40,000	7,800	50,000

*Counts $\times 10^3$.

100 times those obtained by conventional means. It can also be seen that, with a mixed population, in some cases the temperature of the diluent is of critical importance while in other cases it is unimportant. However, in all instances the plate count was highest when phosphate–KCl at 37° C. was used as diluent.

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THE FERMENTATION OF L-ERYTHRULOSE BY AEROBACTER AEROGENES[1]

N. H. Tattrie[2] and A. C. Blackwood[3]

Abstract

L-Erythrulose was dissimilated anaerobically by *Aerobacter aerogenes* PRL R4 producing acetic acid, formic acid, succinic acid, glycolic acid, ethanol, erythritol, hydrogen, carbon dioxide, and traces of acetone and 2,3-butanediol. D-Erythrose and D-threose were also dissimilated to form the same products. Resting cells metabolized the tetroses, whereas cell-free preparations were inactive. Phosphorylation of the tetroses was not found with cell-free preparations.

Introduction

Recent evidence on the importance of erythrose-4-phosphate (12) has stimulated interest in the metabolism of tetroses by microorganisms. In 1900 Bertrand (4) reported that *Acetobacter suboxydans* oxidized erythritol to L-erythrulose. The oxidation and esterification of erythritol by *Propionibacterium pentosaceum* was shown by Barker and Lipmann (3) but a tetrose intermediate was not identified. Kornberg and Racker (13) reported that the enzymatically formed compound is identical with D-erythrose-4-phosphate as synthesized by Ballou *et al.* (1). Hiatt and Horecker (9) studied the metabolism of D-erythrose with strains of *Alcaligenes faecalis* and *Aerobacter aerogenes*. They detected a phosphate ester similar to erythrose-4-phosphate when a cell-free extract of *Alcaligenes faecalis* was incubated with D-erythrose in the presence of adenosine triphosphate (ATP). They showed also that P^{32}-labelled sedoheptulose diphosphate was formed from D-erythrose, sedoheptulose diphosphate, and ATP^{32}, which is further evidence for the formation of D-erythrose-4-phosphate as an intermediate.

Erythrulose is also involved in enzymatic reactions. Charalampous (5) purified an enzyme from rat liver which catalyzed the reversible formation of erythrulose-1-phosphate from formaldehyde and dihydroxyacetone-phosphate. Dickens and Williamson (8) found a yeast carboxylase which formed erythrulose from hydroxypyruvic acid.

In this investigation the products formed by *Aerobacter aerogenes* from a tetrose (sole carbon source) were determined. Resting cell suspensions metabolized L-erythrulose to form ethanol, hydrogen, carbon dioxide, acetic acid, formic acid, succinic acid, glycolic acid, and erythritol with traces of 2,3-butanediol and acetoin.

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Issued as Paper No. 248 on the Uses of Plant Products and as N.R.C. No. 4518.
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Materials and Methods

Fermentation Methods

Aerobacter aerogenes, PRL R4, was grown overnight in a medium containing 0.1% yeast extract, 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% sodium citrate $.2H_2O$, 0.01% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2SO_4$, and 0.2% fructose, as described by Davis and Mingioli (7). The fructose, yeast extract, and mixture of salts were each sterilized separately and combined at the time of inoculation. The cultures were grown aerobically at 30° C. on a rotary shaker in 500 ml. Erlenmeyer flasks containing 200 ml. of the medium. The cells, recovered by centrifugation with aseptic precautions, were washed once with 0.1 *M* $MgSO_4$ and resuspended in 0.1 *M* $MgSO_4$. Two milliliters of the suspension were aseptically transferred to a modified Erlenmeyer flask (14) containing 5 ml. of a sterile suspension of $CaCO_3$ (5 mM.), 4 ml. of a 5% yeast extract solution, 4 ml. of a salt mixture (1% K_2HPO_4, 0.5% $MgSO_4.7H_2O$, 0.5% KCl, and 0.01% $FeSO_4$), 15 ml. substrate (about 5 mM.), and sterile water to give a total volume of 90 ml. The air was then replaced with nitrogen and the fermentation carried out as described by Neish (14).

Preparation of Substrates

D-Threose and D-erythrose were prepared from D-galactose and D-glucose according to the method of Perlin and Brice (17). These compounds were further purified on a cellulose column by elution with 5% aqueous ethyl methyl ketone. Nine grams of D-galactose yielded 3.02 g. D-threose, $[\alpha]_D^{25}$ $-12.7°$ (*c*, 3.8, H_2O). Perlin and Brice (17) reported $[\alpha]_D^{25}$ $-12.9°$ and Hockett (10) reported $[\alpha]_D$ $-12.0°$ for D-threose. Six grams of D-glucose yielded 2.63 g. D-erythrose $[\alpha]_D^{25}$ $-28.2°$ (equilibrium, *c*, 3.2). Perlin and Brice (16) reported $[\alpha]_D^{27}$ $-30.0°$. L-Erythrulose was prepared by the oxidation of erythritol with *Acetobacter suboxydans*, PRL G3. The product was purified on a cellulose column, $[\alpha]_D^{25}$ $+11.8°$ (*c*, 2.1, H_2O). Yield was 6.4 g. of L-erythrulose from 10 g. erythritol. Each of these three tetroses gave a single spot and the same R_f value (0.6) after ascending chromatography on Whatman No. 54 paper in butanol: pyridine: water (5 : 2 : 1.8) when developed with alkaline silver nitrate (21).

Analysis of Fermentation Products

The methods used for the measurement and isolation of the fermentation products were essentially those described by Neish (14).

The amount of substrate fermented was determined by measuring the amount of reducing sugar in the protein-free filtrate by the method of Somogyi (19) and Nelson (15). The reducing power of L-erythrulose was found to be 74% of that obtained by an equal weight of glucose.

Preparation of Cell-free Extracts

Cell-free extracts of *Aerobacter aerogenes* were prepared by the following methods:

1. Thirty milliliters (100 mg. cells/ml.) of a cell suspension in 0.5 *M* phosphate buffer at pH 6.5 was treated in a "Raytheon" 10 kc. sonic oscillator

for 10 minutes at a temperature not higher than 10° C. The sonicate was centrifuged at 4000 × *g* for 15 minutes at 2° C. to remove any remaining whole cells and cell debris.

2. The above procedure was repeated with the same amount of cell suspension in 0.5 *M* phosphate buffer at pH 7.4.

3. Five milliliters of the cell suspension (100 mg. cells/ml.) in 0.5 *M* phosphate buffer at pH 6.5 was treated in the Hughes' press and centrifuged at 4000 × *g* for 15 minutes at 2° C.

4. The foregoing procedure was repeated with addition of 1 g. alumina.

5. Five grams of cells were ground in a mortar for 4 minutes at 2° C. with 10 g. of grinding alumina and extracted with 30 ml. of 0.1 *M* (Tris) 2-amino-2-(hydroxymethyl)-1,3-propanediol–HCl buffer pH 7.4. The mixture was centrifuged at 4000 × *g* for 10 minutes at 2° C.

Results

Fermentation of Tetroses

Aerobic and anaerobic fermentations of 0.2 mM. of L-erythrulose, D-threose, and D-erythrose with whole cell preparations of *Aerobacter aerogenes* for 24 hours indicated that the substrate was utilized, and a compound, chromatographically comparable to erythritol, was formed (Fig. 1). Fermentations

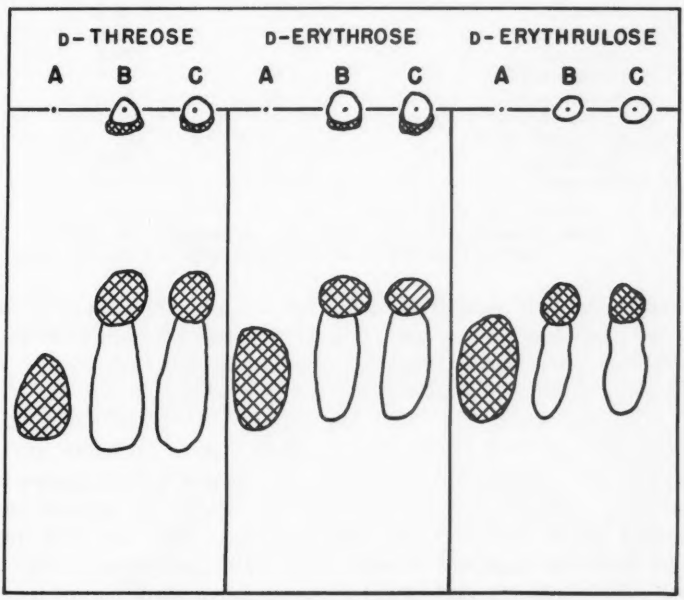

FIG. 1. Schematic tracing of the chromatogram of the fermentation products of tetroses by *Aerobacter aerogenes*.
A. Control.
B. Aerobic fermentation.
C. Anaerobic fermentation.
Solvent: *n*-Butanol : pyridine : water (5 : 2 : 1.8).
Spray : Alkaline silver nitrate.

with larger amounts (5 mM.) of the same substrates were slower and in the majority of experiments no fermentation took place even after 100 hours at 30° C. After several transfers in the tetroses, the organisms did not appear to ferment the substrates at an increased rate; however, L-erythrulose was fermented faster than D-threose or D-erythrose.

The rate of fermentation was determined by aseptically removing small samples of the fermentation mixture with a 0.25 ml. syringe and qualitatively assaying for erythritol by paper chromatography as previously described.

Carbon Balance Studies on L-Erythrulose

The quantities of products obtained from the fermentation of L-erythrulose by *Aerobacter aerogenes* under anaerobic conditions are given in Table I.

TABLE I

PRODUCTS OBTAINED FROM THE DISSIMILATION OF L-ERYTHRULOSE BY *Aerobacter aerogenes*

Product	mM. of product per 100 mM. L-erythrulose dissimilated	mM. of carbon
2,3-Butanediol	0.9	3.6
Acetoin	0.1	0.4
Ethanol	11.4	22.8
Acetic acid	39.8	79.6
Formic acid	1.7	1.7
Succinic acid	6.8	27.2
Carbon dioxide	27.7	27.7
Erythritol	39.9	159.6
Glycolic acid	23.0	46.0
Hydrogen	11.0	—

NOTE: Fermentation time, 96 hours; erythrulose used, 96%; carbon accounted for, 92.2%; O/R balance, 1.12.

The erythritol was isolated from the fermentation mixture by chromatography on a cellulose column using 5% aqueous ethyl methyl ketone. After crystallization from absolute ethanol, the dried product analyzed as: C, 39.58%; H, 8.16%. Calculated for $C_4H_{10}O_4$ (122.12): C, 39.34%; H, 8.25%. Melting point 118°–120° C.; reported 119°–120° C. (11). The compound also gave an infrared spectrum similar to authentic erythritol.

Glycolic acid was identified in the fermentation mixture by paper chromatography using phenol : water : formic acid (75 : 25 : 1) as solvent (20) and bromocresol purple as the developing spray (18). The acid had the same R_f value (0.73) as authentic glycolic acid. The absorption spectra of the acid with 2,7-dihydroxynapthalene in concentrated H_2SO_4 gave a peak at 535 mμ, as did authentic glycolic acid.

Although carbon balances were not completed on D-erythrose and D-threose the same products were formed as with L-erythrulose. Erythritol and glycolic acid were determined by paper chromatography on the protein-free filtrates.

Resting Cell Fermentations

To explore further the pathways by which L-erythrulose may be metabolized, resting cells of *Aerobacter aerogenes* grown on fructose were studied with various substrates in the Warburg apparatus by conventional techniques (22). Cells grown on fructose rapidly metabolized lactate, succinate, glucose, and fructose (Table II) whereas they did not metabolize glycolate and glyoxylate. L-Erythrulose, D-erythrose, and D-threose were oxidized equally while erythritol consistently showed less oxygen consumption (Fig. 2).

TABLE II

OXYGEN UPTAKE WITH RESTING CELL SUSPENSION OF *Aerobacter aerogenes* GROWN ON FRUCTOSE

Substrate	Oxygen consumed (μl.)*	
	1 hour	3 hours
Endogenous	20	40
Fructose	320	572
D-Glucose	310	500
L-Erythrulose	53	95
D-Erythrose	60	105
D-Threose	61	114
Erythritol	51	75
Glycolate	33	45
Glyoxylate	45	65
Lactate	80	225
Succinate	252	343

* Each Warburg cup contained 50 mg. (wet weight) of washed cells; 50 μM. potassium phosphate buffer pH 6.5; 10 μM. substrate, and 0.2 ml. of 10% KOH in the center well. The total volume was 3.2 ml. Incubation temperature was 30° C.

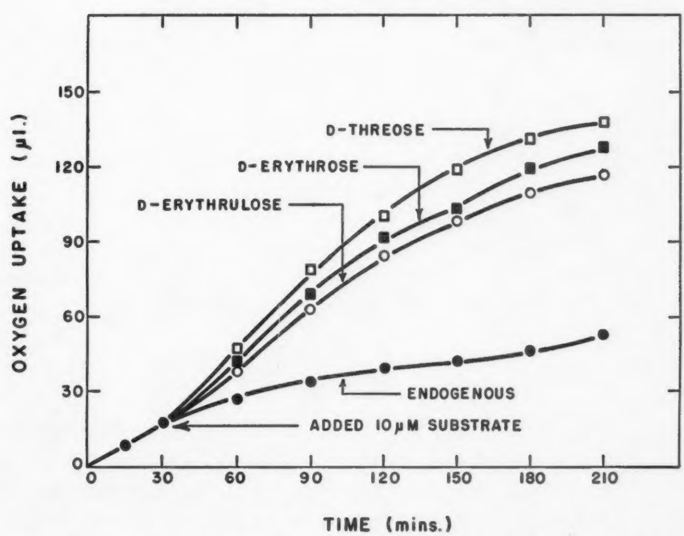

FIG. 2. Each cup contained 45 mg. (wet weight) washed cells; 50 μM. potassium phosphate buffer pH 6.5; 10 μM. substrate; and 0.2 ml. of 10% KOH in center well. Total volume of each cup was 3.2 ml. Temperature was 30° C.

A resting-cell suspension of *Aerobacter aerogenes* grown on fructose and added to 10 μM. of D-threose, D-erythrose, and L-erythrulose consumed 6.7, 4.3, and 3.0 μM. of oxygen in excess of endogenous. During the same period (120 minutes) an excess of 5.4, 3.5, and 1.0 μM. of CO_2 were evolved, giving respiratory quotients of 0.80, 0.81, and 0.33 respectively. These results were repeated and checked several times and no explanation is apparent for the low R.Q. value on L-erythrulose.

Phosphorylation of Tetroses

No demonstrable phosphorylation of D-erythrose, D-threose, L-erythrulose, and erythritol by cell-free extracts of *Aerobacter aerogenes* was observed. Phosphorylation was determined by (*a*) measuring the residual reducing power after precipitation of phosphates with either equal volumes of 0.3 *N* $Ba(OH)_2$ and 5% $ZnSO_4$ or 5 volumes of absolute alcohol and 0.1 volume of 10% barium acetate, and (*b*) by the manometric method of Colowick and Kalckar (6).

In contrast to the results of Hiatt and Horecker (9) no tetrose phosphates were detected by paper chromatography. The cell-free extracts of the tetroses, with and without ATP, were chromatographed, after removal of nucleotides with charcoal, on Whatman No. 54 paper with methanol : formic acid : water (80 : 15 : 5). The chromatograms were sprayed with the Bandurski and Axelrod reagent (2), heated at 85° C. for 5 minutes and treated with H_2S or autoclaved at 8–10 lb. pressure for 2 minutes.

Discussion

Aerobacter aerogenes reduces L-erythrulose to erythritol and apparently D-threose and D-erythrose are likewise reduced. The reduction of D-erythrose to erythritol followed by phosphorylation was suggested by Hiatt and Horecker (9) from the work of Barker and Lipmann (3) with *Propionibacterium pentosaceum.* However, no phosphorylation of erythritol was observed with cell-free extracts of *A. aerogenes.* The presence of glycolate and acetate in the fermentation mixtures of the tetroses suggested that a split between carbons 2 and 3 had taken place.

No explanation of the pathway of the anaerobic dissimilation of L-erythrulose can be made with known enzyme systems. Further research should be carried out with radioactive labelled tetroses to ascertain the metabolic pathway(s) involved.

Acknowledgments

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AMIDOMYCIN, A NEW ANTIBIOTIC FROM A STREPTOMYCES SPECIES. PRODUCTION, ISOLATION, ASSAY, AND BIOLOGICAL PROPERTIES[1]

W. A. TABER AND L. C. VINING

Abstract

Antifungal preparations were obtained from cultures of *Streptomyces* PRL 1642 by solvent extraction of the solids collected after the pH was adjusted to 3.5. The active factor, named amidomycin, was purified by repeated crystallization from aqueous ethanol or petrol (b.p. 60° – 80° C.) to give stable, colorless, optically active needles, m.p. 192° C.

Amidomycin suspended in agar media retarded the growth of many filamentous fungi and noticeably inhibited the plant pathogens *Ustilago maydis*, *Ustilago trebouxii*, as well as the human pathogen, *Hormodendrum pedrosoi*. It also inhibited the germination of uredospores of *Puccinia graminis* at low concentrations. Certain yeasts were completely inhibited by small concentrations of amidomycin; the quantity required was affected by the number of cells in the inoculum.

None of the bacteria examined was inhibited by this antibiotic. At certain concentrations it was lethal to *Candida albicans* as determined by the inability of previously exposed and washed cells to grow on nutrient agar.

Although a few isolated colonies of *Candida albicans* usually developed on plates containing approximately double the concentration required to inhibit growth of the inoculum streak, serial transfer of progeny from such colonies onto agar containing amidomycin did not produce cultures having progressively increasing resistance.

Two degradation products of amidomycin, D(—)-valine and 3,6-diisopropyl-2,5-diketomorpholine, are inactive.

Introduction

Examination of many *Streptomyces* isolated in this laboratory for antagonistic action against *Candida albicans* and other fungi revealed that those having high culture–filtrate activity were invariably producers of antibiotics of the polyene family (8, 16). In an endeavor to isolate an antifungal agent which was not a polyene, those cultures with filtrates showing only limited activity were investigated. Such a culture of *Streptomyces* was isolated from soil collected on the University of Saskatchewan campus. It was found to produce a non-polyene antifungal substance which we have named amidomycin.

Amidomycin resembles valinomycin (3, 4) isolated from a species of *Streptomyces* and the enniatins (5, 10, 11) isolated from species of *Fusarium*, both of which, however, have been reported to inhibit the growth of *Mycobacterium tuberculosis* but have not been reported to inhibit fungi. The structural relationships among these antibiotics have been discussed elsewhere (15).

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Methods

The procedures used in each phase of this study are described under the appropriate headings except for the methods of measuring and evaluating growth, which have been described previously (14).

The Culture

Amidomycin is produced by *Streptomyces* sp. PRL 1642. No attempt was made to identify this *Streptomyces* as to species inasmuch as no up-to-date monograph has been compiled for this genus. It was noted, however, that it could not be identified with any of the cultures described in Waksman's key (18), or Krassilnikov's key (7). In place of a specific epithet, a description patterned in part after that used by Anderson *et al.* (1) and by Benedict *et al.* (2) is presented (Tables I, II, and III). This culture is allocated to Section I of the morphological classification proposed by Pridham *et al.* (12). It conforms to group III*e* in the carbohydrate utilization classification of Zähner and Ettlinger (19). However, it should be noted that variations have been found in the utilization of carbohydrates by isolates of a single culture and by the identical culture when grown on liquid as compared to agar media (9). The carbohydrate utilization classification is based on the performance of agar cultures.

The Inoculum

The inoculum for each experiment except those described under "Isolation" was prepared as follows: Spores from an agar slant culture grown on Waksman's glucose broth (17) supplemented with agar (and hereafter referred to as No. 3 agar medium) were suspended in sterile distilled water and filtered through No. 4 Whatman filter paper using aseptic techniques. The suspension was adjusted to a Klett reading of 85 using filter No. 64. Such a suspension contained approximately 2×10^7 spores/0.1 ml. as determined by haemocytometer count. A fresh suspension was prepared for each experiment. The quantity of this standard inoculum used in each experiment is indicated in the text. The shake cultures used in the isolation of amidomycin were inoculated with 1 ml. of a 48-hour shake culture grown on a medium (YD) consisting of 1% glucose and 1% yeast extract in distilled water and adjusted to pH 7.2 – 7.4. The 5-gallon fermentors were inoculated with 1.5% of a 48-hour YD culture.

The Unit

Crystalline amidomycin suspended in No. 3 agar completely inhibited *Candida albicans* ATCC 10231 at a concentration of 0.6 μg./ml. for up to 3 days. This quantity of the reference batch was assigned the value of 1 unit. One milligram is equivalent to 1667 units.

The Assay

Paper Disk Diffusion Assay; *Assay of Solutions of Amidomycin*

The standard solution was prepared by dissolving 10 mg. amidomycin in 70 ml. ethanol and making to 100 ml. with distilled water. Subsequent

TABLE I

COMPARATIVE GROWTH OF PRL 1642 ON VARIOUS CARBON SOURCES*

Carbon source	Mg. dry mycelium /flask culture	Days incubated	Final pH
None†	16.8 ± 2.3‡	3	6.8
Glucose	153.9 ± 28.7	3	6.4
Galactose	212.7 ± 18.0	3	5.6
Mannose	101.9 ± 13.9	3	6.8
Cellobiose	333.8 ± 25.1	3	5.6
Starch (soluble)	263.1 ± 26.1	3	7.0
Maltose	106.6 ± 21.1	3	6.8
Mannitol	54.2 ± 5.2	3	6.9
Xylose	40.8 ± 2.0	3	6.9
Sucrose	20.1 ± 0.1	3	7.0
Fructose†	13.1 ± 1.5	3	6.7
Ribose	No growth	6	6.4
Cellulose (powdered)	No growth	6	—
Glucose	239.7 ± 15.1	5	4.7
Sorbitol	44.9 ± 1.0	5	7.3
Lactose†	17.8 ± 0.3	5	6.9
Adonitol	Trace	9	6.9
i-Inositol	Trace	9	6.8
Raffinose	Trace	9	6.9
Dulcitol	Trace	9	6.9
L-Rhamnose	Trace	9	—
L-Arabinose	Trace	9	—

* Pridham's medium (for reference see (2)) with agar omitted and asparagine (2 g./liter) added because growth was not satisfactory on inorganic nitrogen. The carbon sources were added to give a concentration equivalent to 15 g. glucose/liter. The carbon sources were autoclaved separately.

† Most of the weight was due to collection of the insoluble salts of the medium on the filter papers used to collect the mycelium.

‡ Mean and standard error of three cultures.

Inoculum: 0.1 ml. of standard spore inoculum.

TABLE II

COMPARATIVE GROWTH OF PRL 1642 ON VARIOUS NITROGEN SOURCES*

Nitrogen source	Days incubated	Mg. dry mycelium /flask culture	Final pH
$(NH_4)_2SO_4$	2	73.1 ± 3.1	6.0
$(NH_4)_2HPO_4$	2	69.6 ± 6.9	6.4
KNO_3	3	24.3 ± 0.4	7.3
Asparagine	3	96.4 ± 14.1	6.9
Glutamate†	2	125.8 ± 1.5	7.9
DL-Valine	3	88.5 ± 1.2	6.3

* Basal medium No. 2-A. This medium was used in place of Pridham's medium because growth was poor on the latter and the insoluble salts interfered with the collection of mycelium for determining dry weights. See Table IV for the composition of this medium. The nitrogen sources which were sterilized separately were added at a concentration giving 0.525 mg. N/ml.

† Adjusted to pH 6.5 before mixture with the basal medium.

Inoculum: 0.1 ml. standard spore inoculum.

TABLE III

CULTURE CHARACTERISTICS OF *Streptomyces* PRL 1642 ON VARIOUS MEDIA*
(5 days)

Medium†	Aerial mycelium	Spore chain and conidiophore	Reverse side of colony	Pigment	Other characteristics
ASA	White, powdery hyphae bearing yellow exudate. Margin entire	Straight and undifferentiated	Brown	Brown and diffusing	
GAA	White, with tufts and margin irregular	Straight and undifferentiated	Pale brown-purple	Pale brown and diffusing	
PA	Raised convoluted moist mycelium bearing patches of white powdery hyphae. Margin entire	Straight and undifferentiated	Cream	No pigment	
DTA	White, powdery hyphae bearing yellow exudate. Margin entire	Straight and undifferentiated	Dark brown	Brown and diffusing	
SA	Moist, colorless mat	—	Cream	No pigment	Starch hydrolysis zone extensive
12% Gelatin in No. 3 agar	White, powdery hyphae. Colony raised and folded above agar surface	—	Dark brown	Extensive brown zone	Gelatin liquefaction
No. 3 Agar	White, powdery hyphae. Margin slightly irregular	—	Dark brown with red tinge	Brown and diffusing. Agar slants bear purple-brown reverse	
Difco litmus milk	White aerial ring	—	—	—	By 5 days dark blue zone (pH 7.5) under colony. Peptonization by 7 days, no decoloration

* Each plate streaked with loop of standard spore inoculum.
† See Anderson *et al.* (1) for composition of first five media. PA refers to their synthetic agar medium and SA to the synthetic medium but containing starch.

dilutions were made in 70% ethanol to give concentrations ranging from 10 to 100 μg./ml. Paper disks dipped in these solutions, drained, and then flicked free of excess liquid were immediately applied to plates of No. 3 agar containing 10 mg. neomycin/liter. Each plate contained 10 ml. unseeded agar and 4 ml. of agar seeded with *Candida albicans* ATCC 10231 at the rate of 5 ml. suspension per 200 ml. agar. The suspension was prepared by mixing in distilled water a 48-hour slant culture grown on No. 3 agar and adjusting to a Klett reading of approximately 260 using filter No. 64. After the paper disks were applied to the plates they were stored at 5° C. for 3 hours to allow diffusion of the antibiotic. They were then incubated for 24 hours at 37° C. Relative potency was determined by the method of Sherwood *et al.* (13). The data were first plotted and parallelism of the curves in a given assay was taken to indicate a valid assay. The constancy of the standard curve slope was examined by use of Knowlden's procedure (6) since the curves of the earlier amidomycin assay, in which impure products and alkali were employed as the diluent, varied considerably in slope. A slope of 6.8 ± 0.3 mm. per log unit was obtained for data from five separate assays. Although fresh standard solutions were used in each assay, it was subsequently found that solutions standing in the dark at 25° C. for 2 months had not lost activity.

Assay of Culture Filtrates

Filtrates could not be conveniently assayed by the above method because of the necessity for using ethanol as a diluent. Earlier studies revealed that filtrates diluted with alkali produced zones. Filtrates were therefore assayed against a standard which consisted of a freeze-dried culture filtrate dissolved in sodium hydroxide. This lot of crude standard was standardized against the reference lot by use of the procedure described above, and was found to contain 27 ± 3 units/mg. This crude standard was dissolved in 0.01 *N* NaOH, and it and the filtrates were diluted with 0.01 *N* NaOH. The standard curve ranged from 1 to 3 mg./ml. Since amidomycin is not soluble in alkali, tests were carried out to determine whether this assay could detect known quantities of amidomycin added to culture filtrates. When 400 units of amidomycin contained in 0.2 ml. of 70% ethanol was made to 10 ml. with a culture filtrate containing 347 units/ml., the total activity was approximately 20% greater than the theoretical value.

Turbidimetric Assay

The assay medium consisted of half-concentration Waksman's glucose broth supplemented with 0.7 g. $NaH_2PO_4.H_2O$ per liter and adjusted to pH 5.5 before autoclaving. Ten milligrams of neomycin were added to each liter after autoclaving. Four and eight-tenths milliliters of sterile medium were added to each sterile, dry Klett tube followed by 0.1 ml. 80% aqueous ethanolic solution of amidomycin, or 0.1 ml. 80% ethanol in the controls, and 0.1 ml. suspension of *Candida albicans*. The inoculum was prepared by growing *Candida albicans* for 20 hours at 25° C. on a rotary shaking machine in a medium consisting of 1% glucose and 1% yeast extract. The cells were

collected by centrifugation, washed once in physiological saline, suspended in saline, and adjusted to a Klett reading of 40. The standard curve ranged from 0.06 to 0.25 μg./ml. The turbidity was read at zero and 24 hours using Klett filter No. 64 and the inhibitory effect expressed as percentage inhibition over the controls after correction for the initial reading. In successive assays 50% inhibition was effected by 0.1, 0.14, 0.1, 0.19, 0.12, and 0.1 μg./ml.

Results

Production

Amidomycin was first produced in shake cultures. One hundred and forty milliliter quantities of a liquid medium consisting of 2% soybean meal and 1% glucose in distilled water, and adjusted to pH 7.2 – 7.4 before autoclaving, were inoculated with 1 ml. of a 48-hour YD culture. These cultures in 500 ml. Erlenmeyer flasks were shaken at 30° C. in the dark on a shaker describing a 2-inch circle and revolving at 110 r.p.m.

Amidomycin is also produced on other media. Concentrations of amidomycin found in filtrates from cultures grown on various media are given in Table IV. It is assumed, on the basis of the parallel curves of the assays, that the active substance in each case was amidomycin. Production was also achieved in this laboratory by use of 5-gal. fermentors but it was irregular and the conditions necessary for constant production are currently being sought.

TABLE IV

AMIDOMYCIN IN FILTRATES OF SHAKEN CULTURES GROWN ON VARIOUS MEDIA*

(Mean and standard error of three cultures. Units/ml. filtrate†)

Medium	Days incubated				
	2	3	4	5	6
1% glucose; 1% yeast extract. pH 7.3	104 ± 30	634 ± 43	497 ± 60	498 ± 31	511 ± 21
1% glucose; 2% soybean meal. pH 7.3	<80	263 ± 31	282 ± 22	272 ± 20	—
1% glucose; 0.5% peptone; 0.5% beef extract; 0.5% NaCl. pH 7.3	—	98 ± 13	151 ± 5	87 ± 5	86 ± 17
Synthetic medium 2A‡	—	—	81 ± 10	—	158 ± 3
Synthetic medium 2A + 140 mg./liter D-valine §	—	—	Trace zone	—	Trace zone
Synthetic medium 2A + 140 mg./liter L-valine §	—	—	Trace zone	—	Trace zone

* Each flask containing 70 ml. of medium was inoculated with 1.0 ml. of standard spore suspension. The cultures were incubated in the dark at 30° C. on a shaker revolving at 110 r.p.m.

† One unit is equivalent to 0.6 μg.

‡ Medium 2A composition is: glucose 25 g.; asparagine 2.5 g.; K_2HPO_4 3.5 g.; KH_2PO_4 1.4 g.; $MgSO_4.7H_2O$ 201 mg.; $CuCl_2.2H_2O$ 0.269 mg.; $CaCl_2.2H_2O$ 5.95 mg.; $FeSO_4.7H_2O$ 5.0 mg.; $ZnSO_4.7H_2O$ 4.40 mg.; $MnSO_4.H_2O$ 2.75 mg.; NaCl 2.55 mg.; $(NH_4)_6Mo_7O_{24}.4H_2O$ 1.82 mg.; distilled water 1 liter. The glucose was autoclaved separately.

§ The effect of valine on production was examined because D-valine occurs in the amidomycin molecule.

Isolation

In a typical experiment the combined mycelium and broth from 3 liters of shaken cultures having a filtrate potency of 276 units/ml. were treated with filter aid (10 g. Hyflo supercel/liter filtrate) and the pH was adjusted to 3.5 with concentrated hydrochloric acid. The solids which were separated by filtration contained all of the activity. Further concentration of the antibiotic was achieved by repeated extraction of the wet residue with methanol, ethanol, or acetone. Methanol was preferred and satisfactory recoveries were achieved by four 1-liter extractions.

The methanol was removed by evaporation *in vacuo* at 40° C. and the residual suspension was diluted with water to 1 liter and freeze-dried. Seven and a half grams of dark brown solid containing 2,400,000 units were obtained. Approximately two-thirds of the activity came from the mycelium. This solid was extracted in a Soxhlet apparatus for 3 to 6 hours with petrol (b.p. 30° – 60° C.). This was evaporated to dryness to give an oily residue which crystallized partially on standing. Repeated recrystallization of this material from petrol (b.p. 60° – 80° C.) or 50% aqueous ethanol gave colorless needles, m.p. 192°C. which contained 1667 units/mg.

The yield of antibiotic recovered could be augmented considerably by chromatography of the mother liquor on silicic acid (Mallinckrodt 2847). The column was prepared as a slurry of silicic acid in benzene and allowed to settle under slight pressure. The dried mother liquors, redissolved in benzene, were applied to the column and developed first with fresh benzene, then with chloroform. Considerable inactive material was removed in the eluates and discarded. Gradient elution with ethanol in chloroform resulted in a sharp zone containing the active material when the amount of ethanol in the developing solvent reached 5%. Evaporation of the solution and recrystallization of the residue as described above yielded amidomycin in a pure state.

Mycelium of cultures, grown in 5-gal. fermentors and harvested following 4 days' incubation, contained little amidomycin and the procedure of isolation was modified as follows: The culture was passed through a Sharples high speed centrifuge and the supernatant liquid collected. After adjustment of the pH to 3.5 with concentrated hydrochloric acid, a flocculent precipitate formed and was collected by a second centrifugation. The precipitate was collected and further purification of the active product was carried out by extraction first into methanol, then into petrol as described above.

Chemical Properties of Amidomycin

Amidomycin is a stable, neutral, optically active ($[\alpha]_D^{25}$ +19.2° (*c*, 1.2, ethanol)) compound, insoluble in water, somewhat soluble in petrol, and very soluble in most organic solvents. The elucidation of its chemical structure (Fig. 1) has been described elsewhere (15). Paper chromatographic examination of the pure substance, as well as crude concentrates and culture filtrates, has shown that only a single active compound of this type is produced

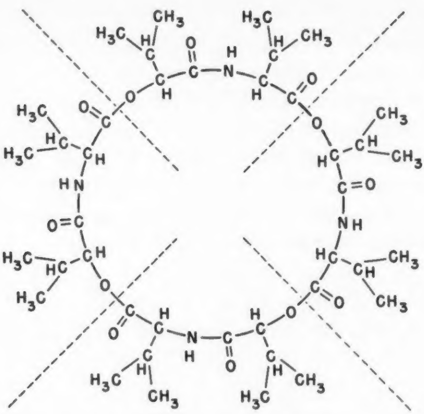

FIG. 1. Chemical structure of amidomycin.

by the strain of *Streptomyces*. The R_f value of amidomycin by descending chromatography on paper impregnated with ethylene glycol with petrol (b.p. 100° – 120° C.) as mobile phase was 0.86. With the solvent systems *n*-amyl alcohol saturated with water, ethyl alcohol – acetic acid – water (3 : 1 : 6), and ethyl alcohol – water (2 : 3), R_f values of 0.90, 0.36, and 0.89 were obtained, respectively. The spots were identified by applying the paper strips to plates of agar seeded with *Candida albicans* and later noting the positions of inhibition.

Amidomycin is very stable. A standard solution stored in the dark at 25° C. for 2 months lost no activity.

Biological Properties

A biological "spectrum" of amidomycin was determined by streaking spore or vegetative cell suspensions from agar slants of various microorganisms over plates of No. 3 agar medium containing different concentrations of amidomycin (Table V). Amidomycin was dissolved in 70% ethanol, diluted with water, and then mixed with the melted agar. Solvent controls were employed. The effect on germination of uredospores of *Puccinia graminis* var. *tritici*, race 15B, was determined by streaking the spores on commercial potato dextrose agar containing various concentrations of amidomycin. Bacterial growth was controlled by the addition of neomycin. Spores exposed to amidomycin developed very short germ tube pegs which never exceeded the length of the spore even when incubated several days. Approximately 66% of the control spores germinated, giving rise to extensive germ tubes.

The concentration required to inhibit the susceptible fungi was affected by the quantity of the inoculum (Table VI). Activity in liquid media was also affected by the size of the inoculum (compare Tables VII and VIII). Two degradation products of amidomycin, D(–)-valine and 3,6-diisopropyl-2,5-diketomorpholine, were inactive.

TABLE V

MICROBIAL SPECTRUM OF AMIDOMYCIN SUSPENDED IN NO. 3 AGAR (28° C.)

PRL No.	Test organism	μg. amidomycin/ml. agar required for complete inhibition	Days
1443	*Candida albicans* (ATCC 10231)	0.6	1*
1439	*Candida pseudotropicalis*	0.6	1
1438	*Candida stellatoidea*	0.7	1
1432	*Candida tropicalis*	5.0	1
1442	*Hormiscium dermatitidis*	0.1	1
	Puccinia graminis var. *tritici* 15B (uredospore germination)	1.5	1
1510	*Pullularia pullulans*	3.2	2
1624	*Ustilago trebouxii*	1.0	3
199	*Ustilago maydis*	2.0	3
1680	*Saccharomyces cerevisiae*	7.0	1†
1434	*Hormodendrum pedrosoi*	7.0	3

1497 *Geotrichum candidum*, 1461 *Geotrichum* sp., 1441 *Geotrichum* sp., 164 *Oidium lactis*, 1516 *Oospora lactis*, 1469 *Aspergillus flavipes*, 1454 *Coniothyrium fuckelii*, 1452 *Isaria cretacea*, 1463 *Penicillium roqueforti*, 1482 *Rhizopus nigricans*, 1435 *Sporotrichum schenkii*, 1492 *Trichothecium roseum*, 1466 *Verticillium albo-atrum*, B44 *Bacillus licheniformis*, R2 *Escherichia coli*, M2 *Micrococcus pyogenes* var. *aureus*, 1681 *Streptomyces endus*, and V5 *Mycobacterium butyricum* were not completely inhibited by 80 μg./ml.

NOTE: 80 μg./ml. was the highest concentration tested.
* 1.0 μg./ml. was required by 4 days but no further changes occurred.
† 60 μg./ml. was required by 10 days.

TABLE VI

EFFECT OF THE CONCENTRATION OF INOCULUM ON AMIDOMYCIN ACTIVITY

(μg./ml. agar required for complete inhibition, 2 days)

Test organism	Inoculum A	Inoculum B	Inoculum C
Candida albicans	>40	12	1.2
Candida stellatoidea	16	2.8	1.2
Saccharomyces cereviseae	>40	>40	12.0
Hormiscium dermatitidis	>40	12	2.8

Inoculum A: Suspension having Klett reading of 500.
Inoculum B: A diluted 1:3.
Inoculum C: A diluted 1:10.
NOTE: One loop of an inoculum was streaked over amidomycin agar.

Fungicidal Property

A fungicidal effect was demonstrable by transferring aliquots of cultures from a turbidity assay to amidomycin-free agar and noting whether or not subsequent growth occurred (Table VII). This viability test is slightly biased toward indicating complete death since the sample consisted of one loop from a 5 ml. culture. Using this criterion, certain concentrations of

TABLE VII

THE COURSE OF INHIBITION AND DEATH IN CULTURES FROM THE TURBIDITY ASSAY*

	Hours incubated											
	None			7			17			24		
Concentration of amidomycin (μg./ml.)	Mean Klett reading	% inhibition	Viability of cells†	Mean Klett reading‡	% inhibition	Viability of cells	Mean Klett reading‡	% inhibition	Viability of cells	Mean Klett reading‡	% inhibition	Viability of cells
7.5	2		++	0.8		+	7.3	93	0	8	94	0
3.8	3		++	0.8		+	7.6	93	0	10	93	0
1.87	1		++	0.3		++	10	90	0	13	91	0
0.94	1.3		++	1		++	16	85	0	13	84	0
0.47	1.6		++	2		++	23	78	0	36	74	0
0.23	0		++	0.3		++	20	80	+	34	75	+
0.12	0.3		++	2		++	45	56	++	72	47	+
0.06	0.3		++	6.5		++	75	26	++	107	20	++
0.03	0		++	9		++	91	10	++	124	8	++
Control	0		++	9		++	101		++	134		++

* At the indicated time the turbidity was read and then a loop was transferred to a No. 3 agar slant.
† ++ = abundant growth.
+ = limited growth.
0 = no growth.
‡ Corrected for initial reading.

TABLE VIII

THE COURSE OF INHIBITION CAUSED BY A SUBLETHAL CONCENTRATION OF AMIDOMYCIN IN THE TURBIDITY ASSAY*

Time of measurement, hr.	Mean Klett reading		% inhibition	Colonies/ml. culture ($\times 10^4$ except at 24 hr., then $\times 10^6$)		
	Control	Amidomycin (suspension) 0.47 μg./ml.		Control	Comparison†	Amidomycin
0	5	7	0	57 ± 4	$t = 2.5$	66 ± 2
3‡	4	0	0	55 ± 7	$t = 0.4$	67 ± 5
8‡	61	48	22	207 ± 13	$t = 4.4$§	123 ± 14
24‡	141	109	23	747 ± 3		10.9 ± 2

* The assay was conducted as described in the text except that a large inoculum (0.1 ml. having Klett reading of 260) was used so that it could be recovered by centrifugation. After the three tubes in each replicate were read they were centrifuged, washed in saline, suspended in saline to the original volume, then plated into melted No. 3 agar.

† A comparison of the plate counts.

‡ Corrected for initial readings.

§ Significant difference ($P < .05$).

amidomycin were concluded to be lethal. If amidomycin is inside the cell in stable, active form, this is equivalent to death. Killing of the cells is not instantaneous. The alternative to the above interpretation is that, given time, amidomycin accumulates on the cell wall and prevents growth when the cells are transferred to another medium. This was tested by plating out cells exposed to a lethal concentration (0.47 μg./ml.) for 3 days after they had been washed three times in one and one-half volumes saline and suspended in saline. Growth did not occur, while the controls treated identically grew in a typical manner. The non-viable, or at least non-proliferating, cells seemed to be identical in appearance with untreated cells. The inhibitory effect of a sublethal concentration of amidomycin was detectable both by turbidity measurements and viable cell counts (Table VIII).

Resistance

Serial transfer of *Candida albicans* onto plates of No. 3 agar containing various concentrations of amidomycin did not lead to a development of increasing resistance. Occasionally, however, isolated colonies would appear on plates in which the inoculum streak was otherwise completely inhibited. Since this might be the result of uneven distribution of amidomycin in the agar rather than to the development of partial resistance, a clone obtained from such a colony was tested for the amount required for 50% inhibition by the turbidity test. The "resistant" line required 0.25 μg./ml. and the parent, 0.11 μg./ml. The dose–response curves were not parallel. The immediate progeny of this line grew well on plates of agar containing slightly more amidomycin than could be tolerated by the parent, but after three transfers on amidomycin-free agar this difference was no longer detectable. Resistance to amidomycin does not appear to be readily induced by exposure to amidomycin.

Acknowledgment

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SOME VITAMIN AND AMINO ACID INTERRELATIONSHIPS IN ESCHERICHIA COLI 113-3

I. THE INHIBITORY EFFECTS OF CYSTINE AND CYSTEINESULPHINIC ACID[1]

J. M. McLaughlan

Abstract

Cystine, cysteinesulphinic acid (CSA), and other closely related sulphur-containing amino acids inhibited growth of *Escherichia coli* 113-3, particularly in aerobic conditions. The cystine inhibition was completely prevented by aspartic acid, partially reversed by pantothenic acid or β-alanine and slightly reversed by lysine or thiamine. The inhibitory effect of CSA was completely or partially reversed by aspartic acid, lysine, glutamic acid, proline, ornithine, or homoserine. Aspartic acid and glutamic acid appeared to reverse the inhibition competitively while lysine seemed to reverse the inhibition in a noncompetitive manner. Reversal of the inhibitory effect of relatively high concentrations of CSA by lysine was not complete, however, unless methionine was also present. Possible mechanisms of the cystine and CSA inhibition are discussed.

Introduction

It has been reported from this laboratory (7) that thiamine depresses the growth response of *Escherichia coli* 113-3 to vitamin B_{12} in aerobic conditions. While testing compounds for possible reversal of the inhibitory effect of thiamine it was found that cystine and related sulphur-containing amino acids also inhibited the aerobic growth of this organism but that thiamine partially reversed the inhibitory effect of cystine.

Several investigators (5, 6, 8, 10, 11, 14) have reported that cystine and cysteine are toxic for various bacteria, particularly when autoclaved with media. It has been shown that colloidal sulphur or metal sulphides, produced during autoclaving of media containing cystine, inhibit growth of *Brucella abortus* (11), *Staphylococcus aureus* (14), and *Mycobacterium tuberculosis* (6). Rowley (10) found, however, that unheated cystine inhibited growth of some strains of *E. coli* and he suggested that cystine interfered with methionine synthesis in the sensitive strains. Dubnoff (5) reported that cysteine and homocystine were toxic for the wild type of *E. coli*. Prince and Cleverdon (8) found that one or more of the following amino acids, histidine, leucine, methionine, proline, and isoleucine reversed the toxicity of cysteine for various species of *Flavobacterium* in simple chemically defined media.

Ravel and Shive (9) reported that cysteic acid, an oxidation product of cysteine, inhibited growth of *E. coli*. The inhibition was reversed competitively by either aspartic acid or glutamic acid and noncompetitively by either β-alanine or pantothenate. They postulated that cysteic acid competitively inhibited the decarboxylation of aspartic acid to give β-alanine, a precursor of pantothenate.

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There do not appear to be any previous reports of an inhibitory effect of cysteinesulphinic acid (CSA) on growth of bacteria. Singer and Kearney (12) and Cobey and Handler (2) have shown, however, that CSA is rapidly metabolized aerobically to pyruvic acid, ammonia, and sulphide by *Proteus vulgaris* and *E. coli.* Schuhardt *et al.* (11) suggested that CSA is formed during autoclaving of cystine solutions. There are several reports in the literature stating that cystine and related sulphur-containing amino acids inhibit growth of bacteria, the toxic effects being attributed to a variety of causes. The purpose of the present work is to compare the inhibitory effects of cystine, cysteine, homocystine, cysteic acid, and CSA for a single organism, *E. coli* 113-3. The mechanisms of the inhibitory effects of cystine and CSA have also been investigated.

Methods

Escherichia coli strain 113-3, which requires either methionine or vitamin B_{12} for growth, was obtained through the courtesy of Dr. B. D. Davis of the Department of Pharmacology, New York University. In most tests a variant of *E. coli* 113-3, requiring approximately 0.018 mμg. of vitamin B_{12} per ml. for half-maximal growth, was used since it appeared to be more sensitive to cystine than the original culture. *E. coli* 113-3 was maintained and assays were done as described previously (7). Unless otherwise stated, all cultures were shaken during growth and both stationary and shaken cultures were incubated for 16 hours at 31°. The minimal medium was that of Davis and Mingioli (4). It contained: 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% Na_3-citrate.$3H_2O$, 0.01% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2SO_4$, and 0.2% dextrose (autoclaved separately). The medium was supplemented with sufficient vitamin B_{12} (0.1 mμg./ml.) or DL-methionine (10 μg./ml.) for optimal growth.

The effects of the sulphur-containing amino acids were tested at several growth limiting, as well as at optimal, concentrations of vitamin B_{12} or methionine. Only the data from tests with optimal amounts of vitamin B_{12} or methionine, however, are reported. CSA was sterilized by filtration through sintered glass or it was "flash pasteurized" (95° C. for 1 minute) before it was added aseptically to tubes. The other sulphur-containing amino acids were autoclaved for 3 minutes with the medium, unless otherwise stated.

Results

The relative inhibitory effects of cystine and related sulphur-containing amino acids on the growth of *E. coli* 113-3 in media containing vitamin B_{12} or methionine are shown in Table I. All compounds tested inhibited aerobic growth although cystine and CSA appeared to be the most toxic. These substances were less inhibitory in semiaerobic conditions (stationary tubes) and cystine actually stimulated growth in media containing suboptimal concentrations of vitamin B_{12}. The inhibitory effects actually resulted from a prolongation of the lag period of growth since the degree of inhibition diminished when cultures were incubated longer than 16 hours.

TABLE I

THE EFFECT OF SULPHUR CONTAINING AMINO ACIDS ON GROWTH OF *E. coli* 113-3

Amino acid (μg./ml.)		Amount of growth*	
		Aerobic	Semiaerobic
L-Cysteic acid	30	++	+++
DL-Homocystine	10	++	+++
L-Cysteine	10	+	+++
L-Cystine	10	−	++++
L-Cysteinesulphinic acid	2.5	−	+++

*++++ Denotes full growth.
+++ Denotes nearly full growth.
++ Denotes one half-maximal growth.
+ Denotes slight growth.
− Denotes no growth.

The effects of several concentrations of cystine on the growth of *E. coli* 113-3 are shown in Table II. The inhibitory effect increased with increasing amounts of cystine and 5 μg. per ml. almost completely inhibited growth. A sterile, unheated solution of cystine added aseptically to tubes appeared to be somewhat less inhibitory than cystine autoclaved with the medium.

Various compounds were tested for ability to reverse the cystine inhibition and the results compiled from several tests are given in Table III. Thiamine had a slight but definite reversing effect over a wide range of concentrations of both thiamine and cystine. Pantothenate was more effective than thiamine and the extent of reversal by pantothenate appeared to be about the same for heated or for unheated cystine. The effect of β-alanine was similar to that of pantothenate and both compounds seemed to be more effective in media

TABLE II

THE EFFECT OF CYSTINE CONCENTRATION ON THE GROWTH OF *E. coli* 113-3

Amount of cystine (μg./ml.)	Absorbance*	
	Expt. 1	Expt. 2
0	1.19	1.15
1.25	0.60	—
2.5	0.28	—
5.0	0.11	—
10.0	0.09	0.02
10.0†	—	0.15

* A measure of culture turbidity (2-log galvanometer reading).
† A sterile, unheated solution of cystine was added aseptically to the autoclaved medium after cooling.
NOTE: The medium contained methionine.

containing methionine than in media with vitamin B_{12}. Aspartic acid completely reversed the cystine inhibition but lysine was only slightly effective. Pantothenate and lysine produced approximately the same degree of reversal as pantothenate alone.

The CSA inhibition was more marked than the cystine inhibition; as little as 2.5 μg. per ml. completely inhibited growth. Amino acids were tested singly for ability to reverse the effect of CSA; the data are given in Table IV.

TABLE III

REVERSAL OF THE CYSTINE INHIBITION BY COMPOUNDS RELATED TO PANTOTHENIC ACID METABOLISM

Compound added (μg./ml.)		Amount of growth in media containing*	
		Vitamin B_{12}	Methionine
None		−	−
Thiamine (B_1)	0.1	+	+
Pantothenic acid (PA)	0.15	++	+++†
B_1 + PA		++	+++
β-Alanine	2.0	++	+++
DL-Aspartic acid	20.0	++++	++++
L-Lysine	10.0		+
Lysine + PA			+++

* See footnotes Table I.
† With autoclaved or aseptically added cystine.
NOTE: The medium contained 10 μg. of cystine per ml.

TABLE IV

REVERSAL OF THE INHIBITORY EFFECT OF CYSTEINESULPHINIC ACID ON THE GROWTH OF *E. coli* 113-3 BY AMINO ACIDS AND PANTOTHENATE

Amino acid added*	Amount of growth in media containing†	
	Vitamin B_{12}	Methionine
None	−	−
DL-Aspartic acid	++++	++++
DL-Homoserine	+	+
DL-Threonine	−	−
DL-Methionine	−	−
L-Lysine	+++	++++
β-Alanine	+	+
Ca-D-pantothenate	−	−
DL-Glutamic acid	++++	++++
L-Proline	+++	++
DL-Ornithine	++	−
L-Hydroxyproline	+	−
L-Arginine	−	−

* Amino acids were tested at a concentration of 5 μg. of the L-enantiomorph per ml. of medium; Ca-D-pantothenate was tested at a concentration of 0.1 μg. per ml. of medium.
† See footnotes Table I.
NOTE: All tubes contained 5 μg. of CSA per ml. of medium.

As with the cystine inhibition aspartic acid completely reversed the effect of CSA, but pantothenate had little or no reversing effect and β-alanine was only slightly effective. Homoserine reversed the toxicity of CSA slightly but threonine and methionine were ineffective. Lysine completely reversed the CSA inhibition in media with methionine but was less effective in the medium containing vitamin B_{12}. Proline, ornithine, and hydroxyproline, however, appeared to be slightly more effective when the medium contained vitamin B_{12}. Other amino acids which had little or no reversing effect were: isoleucine, histidine, cysteine, phenylalanine, tyrosine, serine, valine, α-alanine, and glycine.

The reversing effects of aspartic acid and lysine were tested over a wide range of concentrations and some of the data are given in Table V. In this experiment the medium contained an optimal amount of methionine for growth. The data indicated that aspartic acid competitively reversed the CSA inhibition whereas lysine reversed it in a noncompetitive manner. In other tests (data not shown) glutamic acid also appeared to reverse the CSA inhibition competitively.

TABLE V

REVERSAL OF THE INHIBITORY EFFECT OF CYSTEINESULPHINIC ACID ON THE GROWTH OF *E. coli* 113-3 BY ASPARTIC ACID AND LYSINE

L-Cysteinesulphinic acid (μg./ml.)	Reversing agent			
	L-Aspartic acid		L-Lysine	
	μg./ml.	Absorbance*	μg./ml.	Absorbance*
0	0	1.19	—	—
0	400	1.22	20	1.22
5	0	0.01	—	—
10	2	0.07	—	—
10	5	1.22	—	—
20	2	0.12	—	—
20	10	1.16	—	—
100	10	0.21	10	1.16
100	100	1.22	20	1.16
200	20	0.27	10	1.16
200	200	1.22	20	1.16
400	40	0.37	10	1.13
400	400	1.22	20	1.13

* See footnote Table II.
NOTE: The basal medium contained methionine.

Several tests indicated that lysine only partially reversed the inhibitory effect of high concentrations of CSA when the medium contained vitamin B_{12} instead of methionine. Several combinations of amino acids were tested for ability to reverse the effect of relatively high concentrations of CSA and the data are given in Table VI. In both experiments, lysine produced only partial reversal of the CSA inhibition in the absence of methionine. Homoserine in the absence of CSA inhibited growth to some extent, making it difficult to evaluate results with homoserine. In Experiment 2 methionine

plus threonine had no reversing effect but lysine with methionine, or lysine with methionine plus threonine, produced complete reversal of the CSA inhibition.

TABLE VI

REVERSAL OF THE INHIBITORY EFFECT OF CYSTEINESULPHINIC ACID ON THE GROWTH OF *E. coli* 113-3 BY COMBINATIONS OF AMINO ACIDS

Additions to the basal medium*						Absorbance†	
Vitamin B_{12}	Methionine	CSA	Homoserine	Threonine	Lysine	Expt. 1	Expt. 2
+	−	−	−	−	−	1.35	0.96
−	+	−	−	−	−	1.30	−
+	−	+	−	−	−	0.03	0.02
−	+	+	−	−	−	0.02	−
+	+	+	−	−	−	−	0.02
+	−	+	−	−	+	0.68	0.77
−	+	+	−	−	+	1.03	−
+	−	−	+	−	−	0.48	−
+	−	+	+	−	−	0.04	−
+	−	+	+	−	+	0.29	−
+	+	+	−	−	+	−	1.10
+	+	+	−	+	−	−	0.03
+	+	−	−	+	+	−	1.22
+	+	+	−	+	+	−	1.22

* A positive sign denotes that the compound was added to medium. The amounts of amino acids per ml. of medium were: DL-methionine, 20 μg. and 10 μg. for Expts. 1 and 2 respectively; L-lysine, 20 μg. and 10 μg. for Expts. 1 and 2 respectively; DL-homoserine, 40 μg.; DL-threonine, 5.0 μg.; CSA, 200 μg. and 20 μg. for Expts. 1 and 2 respectively.

† See footnote Table II.

Discussion

Cystine and the related sulphur-containing amino acids were less toxic in semiaerobic than in aerobic conditions; however, the reason for this difference is not apparent. It is possible that *E. coli* 113-3 reduces these compounds to noninhibitory substances under semiaerobic conditions. CSA appeared to be the most toxic and cysteic acid seemed to be the least toxic for *E. coli* 113-3. It is probable that cysteine is partially oxidized to cystine in aerobic conditions and the apparent effect of cysteine may actually be due to cystine.

The metabolism of *E. coli* is altered when methionine is added to the medium since the "methionine synthase system" (3) is inoperative. It is not surprising, therefore, that the inhibitory effects of cystine and of CSA (and the reversal of these inhibitions by other compounds) differed when the medium contained methionine instead of vitamin B_{12}. As discussed below this difference may possibly be explained with CSA, but the reason for the difference is not apparent with cystine.

Schuhardt *et al.* (11) suggested that CSA was one of the products formed during autoclaving of cystine solutions. When CSA was found to inhibit growth of *E. coli* 113-3 it seemed probable that CSA was responsible for the apparent inhibitory effect of cystine; this hypothesis was strengthened by the finding that aspartic acid reversed both the cystine and CSA inhibitions. It is clear, however, that different mechanisms of inhibition are involved.

Pantothenate partially reversed the cystine inhibition but it had little or no effect on the CSA inhibition. Lysine reversed the toxicity of CSA but it had only a slight effect on the cystine inhibition. Moreover, unheated cystine also inhibited growth and the inhibition was partially reversed by pantothenate. If toxic degradation products of cystine were produced during the relatively short autoclaving period, these undoubtedly contributed to the apparent inhibitory effect of autoclaved cystine. The cystine inhibition closely resembled the cysteic acid inhibition (9) since it was completely reversed by aspartic acid and partially reversed by β-alanine or panthothenate. It is unlikely, however, that the toxic effect of cystine resulted from oxidation of cystine to cysteic acid since cysteic acid was considerably less toxic than cystine for *E. coli* 113-3. Nevertheless, cystine like cysteic acid appeared to interfere with the synthesis of pantothenate from aspartic acid.

Ravel and Shive (9) found that either glutamic acid or aspartic acid competitively reversed the cysteic acid inhibition with *E. coli.* Their studies indicated that a very rapid transamination with oxalacetic acid resulted in the formation of essentially equivalent amounts of aspartic acid from supplementary glutamic acid. Likewise in the present work, glutamic acid appeared to reverse the CSA inhibition competitively. It is probable therefore that glutamic acid, in this instance also, was acting simply as a precursor of aspartic acid. Glutamic acid is a direct precursor of proline and ornithine (13) and the reversal of the CSA inhibition by these compounds may be due to a sparing effect on glutamic acid or to the reversal of the reactions producing these compounds from glutamic acid.

Aspartic acid has been shown (1) to be direct precursor of homoserine, methionine, threonine, and lysine. Homoserine at relatively low concentrations appeared to antagonize the action of CSA, but higher concentrations of homoserine inhibited growth. Neither methionine nor threonine were effective. Aspartic acid reversed the CSA inhibition competitively while lysine appeared to reverse it noncompetitively. Diamino pimelic acid, the apparent immediate precursor (15) of lysine was not available for testing.

From the studies it would appear that the primary effect of CSA is to inhibit competitively the utilization of aspartic acid (or a closely related compound) for lysine synthesis. Although methionine per se had no effect on the CSA inhibition, lysine produced complete reversal of the inhibitory effects of relatively high concentrations of CSA only when the medium contained methionine. This might imply that the primary site of the CSA inhibition is the conversion of aspartic acid to lysine but that a secondary system affected is the synthesis of methionine from aspartic acid.

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STUDIES ON THE ANTIGENIC STRUCTURE OF HISTOPLASMA CAPSULATUM[1]

N. A. Labzoffsky, J. B. Fischer, and J. J. Hamvas

Abstract

Employing physical and chemical methods eight antigenic fractions were isolated from *Histoplasma capsulatum* as determined by complement fixation technique. Two of the fractions were found to cross-react with coccidioidal antisera, two with coccidioidal and *Blastomyces* antisera, one with *Blastomyces* antisera, while the remaining three displayed specificity by reacting with *Histoplasma* antisera only. Some evidence is presented to indicate that the isolated fractions are antigenically distinct.

Introduction

Preliminary work on the antigenic structure of *Histoplasma capsulatum* revealed the presence of at least three antigenically distinct fractions. Further, antibodies, both in humans as well as in experimental animals, for each fraction appear at different times during the course of illness (5).

This work has been extended further and the findings are reported in this communication.

Materials and Methods

Culture

The same strain of *H. capsulatum* No. 467 used in previous work was employed in this study and was grown in the same manner (5, 6).

Procedures of Fractionation

The fractionation steps are outlined diagrammatically in Fig. 1. All steps were carried out at RT° unless otherwise noted. Sterile technique was employed as far as practicable.

Reagents: pyridine = laboratory reagent
urea = urea crystal "Baker Analysed" reagent
acetone = Commercial
phenol = Phenol U.S.P. fused crystals
10% trichloracetic acid
phosphate buffer 0.05 *M* pH 7.2

Step 1.—has been described previously in preparation of the crude antigen (6). Briefly the procedure consisted of treating washed sediment of the yeast phase cells with 20 times its volume of pyridine for 2 hours, collecting the pyridine extract and washing the cells with distilled water three times, and resuspending them after final washing in distilled water 1:20 by volume.

Step 2.—Pyridine extract from Step 1 was diluted 1:2 with distilled water, transferred to a dialyzing bag which then was half-submerged in a container of distilled water placed in front of an electric fan. The material was dialyzed and pervaporated until the volume was reduced to approximately one-third.

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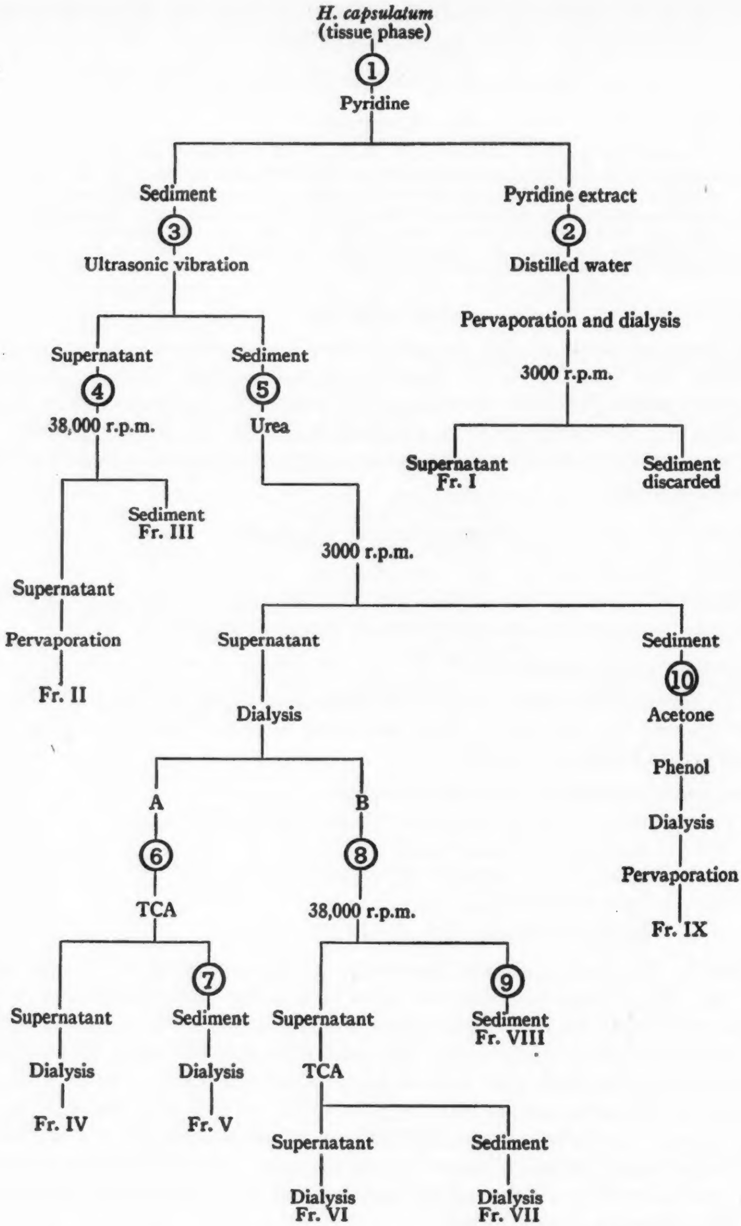

FIG. 1. Flow diagram of basic procedures in fractionation of *H. capsulatum*.

During this process the distilled water was changed three to four times. Next the extract was dialyzed against tap water for 24 hours and further pervaporated in front of a fan to about one-tenth of its original volume. Finally the concentrated material was dialyzed 24 hours in each case, against tap and then distilled water, centrifuged for 20 minutes at 3000 r.p.m., the supernatant collected and designated as Fraction I. The sediment was found to be not antigenic and was discarded.

Step 3.—Resuspended yeast phase cells from Step 1 were exposed to ultrasonic vibration (ultrasonic generator and vessels used were described earlier (4, 6)), for several periods of 2 hours each. After each period of vibration the material was centrifuged at 3000 r.p.m. for 20 minutes, the supernatant collected, and the sediment resuspended in distilled water to the original volume. The cycle of vibration was repeated until the last supernatant showed negligible antigenicity when tested by complement fixation. The sediment, after the last vibration, was saved for further treatment as described below in Step 5.

Step 4.—The supernatants were pooled and centrifuged at 38,000 r.p.m. for 90 minutes. The resultant supernatant was placed in a dialyzing sac and pervaporated in front of a fan to approximately one-twentieth of its original volume, dialyzed 24 hours against distilled water and then 24 hours against 0.85% sodium chloride. The product was designated Fraction II. The sediment remaining after centrifugation at 38,000 r.p.m. was marked Fraction III.

Step 5.—The sediment from Step 3 was treated with urea crystals using with some modification, the procedure of Seibert and Fabrizio (8). The wet sediment was mixed with an equal weight of urea crystals and left in a water bath at 37° C. for 72 hours with an occasional shaking. At the end of this period approximately 2 volumes of distilled water were added and after thorough mixing the material was returned to the water bath for an additional 24 hours. During this period the material was periodically shaken. Next, it was centrifuged at 3000 r.p.m. for 30 minutes, the extract collected, and the sediment washed three times with distilled water. Since these washings were found to contain a considerable amount of protein and were antigenic, they were pooled and added to the original extract. The sediment was re-extracted with urea once more, following the same procedure. Both extracts were pooled and the sediment saved for further treatment to be described in Step 8. The pooled extract was dialyzed against tap water for 24 hours and divided into two parts.

Step 6.—To one part of the dialyzed extract sufficient 10% trichloracetic acid (TCA) was added to bring about turbidity (final TCA concentration around 3%) and the mixture left in 37° C. water bath for 15 minutes. It was then centrifuged. The sediment was set aside for further treatment, Step 7. The supernatant was successively dialyzed against tap water, distilled water, and distilled water buffered at pH 7.2, each for 24 hours. The product thus obtained was designated Fraction IV.

Step 7.—The sediment obtained by TCA precipitation in Step 6 was redissolved in distilled water buffered at pH 7.2, reprecipitated with TCA three more times, dialyzed against tap water, distilled water, and buffered distilled water each for 24 hours. This product was designated Fraction V.

Step 8.—The second part of the dialyzed urea extract (Step 5) was centrifuged at 38,000 r.p.m. for 90 minutes. The supernatant was treated with TCA as in Step 6 and centrifuged. The resulting supernatant was dialyzed against tap water, distilled water, and buffered distilled water and designated Fraction VI. The material precipitated by the TCA was redissolved in buffer, dialyzed as above, and designated Fraction VII.

Step 9.—The sediment produced by centrifugation at 38,000 r.p.m. of urea extract (Step 8) was redissolved in buffered distilled water, dialyzed, and designated Fraction VIII.

Step 10.—The residue from the urea extraction (Step 5) was again washed three times with distilled water and the polysaccharide extracted by a modification of Palmer's method (7). To the centrifuged sediment five volumes of cold acetone were added and mixed thoroughly. After 30 minutes of contact the mixture was centrifuged, the acetone extract removed, and the extraction repeated twice. The acetone was evaporated from the residue by heating in a water bath at 58° C. for approximately 2 hours. The solid residue was then pulverized with the aid of glass beads. To the dried powder phenol crystals were added (approximately twice the volume of dry residue) and the material was kept in the water bath at 58° C. for 30 minutes with an occasional stirring. Next, five volumes of cold distilled water were added to the phenol suspension and the material was centrifuged at 3000 r.p.m. for 30 minutes. The collected supernatant was dialyzed against tap water for 24 hours and against distilled water for an additional 24 hours and finally it was pervaporated to 1/10th of its volume. The last two operations were performed at 4° C. The final product was tested for the presence of protein and if detected, the protein was precipitated with trichloracetic acid and the cycle of dialysis repeated. The final product was designated as Fraction IX.

Analytical Procedures

A number of spot tests were performed on each fraction for protein and carbohydrate determinations. For demonstration of arginine, the Sakaguchi test was employed using one drop of 0.3% α-naphthol in three drops of the solution to be tested and oxidized by bromine water. Tryptophan was tested by *p*-dimethylamino-benzaldehyde in the presence of 20% hydrochloric acid (1). This was further verified by the Adamkiewicz test for tryptophan. Ninhydrin test was performed according to Feigl's procedure (2). Standard lead acetate method was used for detection of cysteine (1% lead acetate and 10% NaOH). As a general test for proteins the xanthoproteic test was employed.

Reducing sugars were identified by the triphenyl-tetrazolium-chloride test according to Feigl (3). Polysaccharide content was determined by the

Molisch test using 1% α-naphthol solution. Pentoses were tested with the orcinol–HCl–$FeCl_3$ reaction (Bial's) modified to dropsize quantities.

Absorption Spectra

Measurements were carried out in Beckman Model DU Spectrophotometer with the continuous spectrum of the hydrogen discharge tube. All fractions were diluted to contain 5 antigenic units per cc. and the pH in all cases was adjusted to 6.4.

Complement Fixation Test

The technique of the complement fixation test was described earlier (4, 6). All the usual controls were always included in each test, but for the sake of brevity were omitted from the tables in the text. Antigenic unit was determined in each case by checkerboard titration of the fraction against the same standard immune serum.

Sera

Rabbit and human antisera were used in testing various fractions.

Immune rabbit sera were used in evaluation of the antigenicity of different fractions. The sera were prepared as described earlier (6). Trial samples of sera were collected at weekly intervals during the course of immunization. In the final analysis all trial samples of serum were titrated simultaneously against all the fractions, each of which was diluted to contain two antigenic units. For species specificity each fraction was tested against *Blastomyces* and coccidioidal antisera. *Blastomyces* antisera were prepared in rabbits using the same procedure of immunization as that for *Histoplasma* antiserum (2). Coccidioidal antisera were kindly supplied by Dr. C. E. Smith of University of California from human cases in various stages of infection.

Experimental Results

All nine fractions obtained were soluble with the exception of Fraction III, which was particulate, and all were found to be heat stable. With the exception of Fraction VII all were found to be antigenically active. Results of the titration of these fractions are summarized in Table I.

TABLE I

RESULTS OF THE TITRATION OF *H. capsulatum* FRACTIONS AGAINST IMMUNE RABBIT SERUM

	Antigen 1 in							
	16	32	64	128	256	512	1024	2048
Fraction I	++++	++++	++++	++++	++++	++++	++++	+++
Fraction II	++++	++++	++++	++++	++++	++++	++	+
Fraction III	++++	++++	++++	++++	++++	++++	++++	+++
Fraction IV	++++	+++	−	−				
Fraction V	++++	++++	++++	++++	++++	++	−	−
Fraction VI	++++	+++	−	−				
Fraction VII	−	−	−	−				
Fraction VIII	++++	++++	++++	++++	++++	++++	+++	++
Fraction IX	++++	++++	++++	+++	−	−		

Immune serum 1:40.

As is seen from Table I all fractions except VII exhibited a considerable degree of antigenicity. Differences in titer of the various fractions are not indicative of their relative proportions in the original material but rather of the degree of concentration in their preparation. All these fractions were found to be stable for at least several months.

To differentiate these fractions several serological tests were performed.

In Table II results of the simultaneous titration of *Histoplasma* antisera from weekly trial bleedings against various fractions are tabulated.

As shown in Table II, serum sample from the second week's bleeding indicates that antibodies for Fractions V and IX appear first. It is regrettable that bleeding at the first week after infection was not made for it might have differentiated between these two fractions. Subsequent titration, however, of the serum taken at the second week showed that its titer against Fraction IX was 1:64, while its titer against Fraction V was 1:256. This fourfold

TABLE II

TITRATION OF TRIAL BLEEDINGS FROM RABBIT NO. 926 AGAINST FRACTIONS OF *H. capsulatum*

		Serum 1 in			
		8	16	32	64
Bled 2 weeks after infection	Fr. I	−	−	−	−
	Fr. II	−	−	−	−
	Fr. III	+++	+	−	−
	Fr. IV	−	−	−	−
	Fr. V	++++	++++	++++	++++
	Fr. VI	−	−	−	−
	Fr. VIII	−	−	−	−
	Fr. IX	++++	++++	++++	+++
Bled 3 weeks after infection	Fr. I	++++	−	−	−
	Fr. II	++++	+++	++	−
	Fr. III	++++	++++	++++	+++
	Fr. IV	−	−	−	−
	Fr. V	++++	++++	++++	++++
	Fr. VI	++	−	−	−
	Fr. VIII	−	−	−	−
	Fr. IX	++++	++++	++++	++++
Bled 4 weeks after infection	Fr. I	++++	++++	+++	++
	Fr. II	++++	++++	+++	+
	Fr. III	++++	++++	++++	++++
	Fr. IV	++++	++	−	−
	Fr. V	++++	++++	++++	++++
	Fr. VI	++++	++++	++	−
	Fr. VIII	++++	+++	+	−
	Fr. IX	++++	++++	++++	++++
Bled 5 weeks after infection	Fr. I	++++	++++	++++	++++
	Fr. II	++++	++++	++++	+++
	Fr. III	++++	++++	++++	++++
	Fr. IV	++++	+++	++	−
	Fr. V	++++	++++	++++	++++
	Fr. VI	++++	++++	++++	+++
	Fr. VIII	++++	++++	++++	++++
	Fr. IX	++++	++++	++++	++++

difference in titer may be interpreted as due to antigenic difference between the two fractions. The data presented in Table II would seem to indicate that antibodies for the various fractions appear roughly in the following order: V, IX, III, II, I, VI, VIII, and IV. Although the data here presented do not prove conclusively that each of the fractions is antigenically distinct, the appearance of antibodies to the various fractions at different times suggests that some, at least, of the fractions are antigenically unrelated. This will be discussed more fully later.

Unfortunately owing to an epizootic in the animal colony it was possible to prepare antisera against only Fractions I and VIII. These sera were tested against all the fractions and the results obtained, as shown in Table III, indicate that at least Fractions I and VIII are antigenically distinct.

TABLE III

RESULTS OF COMPLEMENT FIXATION TEST OF FRACTIONS I AND VIII ANTISERA AGAINST FRACTIONS OF *H. capsulatum*

	H. capsulatum fraction							
	I	II	III	IV	V	VI	VIII	IX
Rabbit No. 223 1:8	–	–	–	–	–	–	++++	–
Rabbit No. 224 1:8	++++	–	–	–	–	–	–	–

Rabbit No. 223 immunized with Fr. VIII.
Rabbit No. 224 immunized with Fr. I.

Further attempt has been made to differentiate these fractions by testing them against coccidioidal and *Blastomyces* antisera and the protocol of this test is given in Table IV. Dilutions of coccidioidal antisera used in this test were the lowest dilutions which were found to be not anticomplementary.

Table IV shows that all antisera (*Histoplasma*, *Blastomyces*, and coccidioidal) reacted, as might be expected, with crude *Histoplasma* antigen and the same was true with Fraction III, indicating that this fraction is common to all three organisms. In addition *Blastomyces* antiserum reacted with Fractions I and II. This latter observation was confirmed with other *Blastomyces* antisera and here it was noted that antibodies for Fractions I and II appeared later than that for Fraction III.

The reactions between some of the fractions and coccidioidal antisera present a somewhat confusing picture. As mentioned before, Fraction III reacted with all coccidioidal antisera. Fraction I reacted only with two of these sera (No. 4 and No. 5) and Fractions V and VIII with only one (No. 5). According to Dr. C. E. Smith, the patient from whom serum 5 was obtained had hemoptysis in 1952 and was discovered to have a thin walled cavity in his lung. He was tuberculin negative but reacted to coccidioidin. He has continued to have a high titer of complement-fixing antibodies against *Coccidioides* from 1953 to 1956. It is of course possible that the patient had had histoplasmosis in the past in which case the residual antibodies would be responsible for this picture. However, no proof of this is available and no histoplasmin test was done on the patient.

TABLE IV

RESULTS OF THE TEST OF VARIOUS FRACTIONS OF *H. capsulatum* AGAINST COCCIDIOIDAL, *Blastomyces*, AND *Histoplasma* ANTISERUM

	Coccidioidal antisera No.									*Blastomyces* antiserum	*Histoplasma* antiserum
	1	2	3	4	5	6	7	8	9		
	Serum dilution tested										
	4	4	6	4	6	6	4	4	4	8	40
Fraction I	−	−	−	++++	++++	−	−	−	−	++++	++++
Fraction II	−	−	−	−	−	−	−	−	−	++++	++++
Fraction III	++++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Fraction IV	−	−	−	−	−	−	−	−	−	−	++++
Fraction V	−	−	−	−	++++	−	−	−	−	−	++++
Fraction VI	−	−	−	−	−	−	−	−	−	−	++++
Fraction VIII	−	−	−	−	++++	−	−	−	−	−	++++
Fraction IX	−	−	−	−	−	−	−	−	−	−	++++
Crude *Histoplasma* antigen	++++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++

TABLE V

CHEMICAL ANALYSIS OF THE FRACTIONS OBTAINED FROM *H. capsulatum*

Antigen fractions	Sakaguchi (arginine) test	Tryptophan test	Adamkiewicz test	Ninhydrin test	Cystine test	Xanthoprotein test	Reducing sugars	Molisch test	Bial's (pentose) test
I	+	+	+	+	Traces	+	++	++	−
II	+	++	+	++	−	+++	++	+	−
III	+	++	+++	++	+	+++	++	+	++
IV	++	++	+	++	−	+	+	+	+
V	++	+	++	+	−	+	++	+	++
VI	+	+	+	+	−	+	+	+	+
VIII	+	+	++	+	Traces	+	+	+	+
IX	−	−	−	+	−	Traces	+++	+++	++

The results here presented do not differentiate between Fractions IV, VI, and IX but do indicate that they are all specific for *Histoplasma capsulatum* and could be used to distinguish between *Histoplasma* antiserum and antisera for *Blastomyces* and *Coccidioides.*

An attempt has also been made to differentiate these fractions chemically and also with the aid of ultraviolet absorption tests. The results of chemical analysis are recorded in Table V and those of ultraviolet absorption tests in Fig. 2.

Although no dramatic differences were noted among the fractions with the chemical tests employed yet certain differentiations could be made as is seen in Table V. Fraction IX is distinct from the other fractions as demonstrated by the tests for proteins. According to Bial's test, Fractions I and II differ from the rest of the fractions. Test for cystine and Adamkiewicz test differentiate Fraction III from the others. Xanthoprotein test to some degree differentiates Fraction I from Fraction II.

Although no significant difference is noted between Fractions IV, V, VI, and VIII in Table V these fractions appear to be different on spectrophotometric analysis as shown in Fig. 2. Thus Fraction IV differs from Fractions

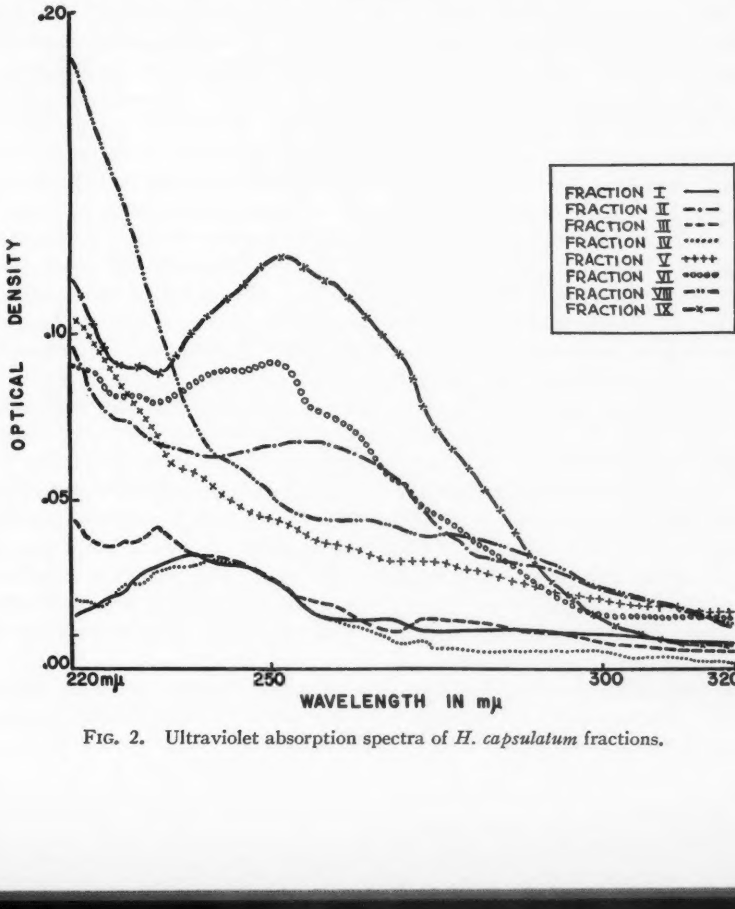

FIG. 2. Ultraviolet absorption spectra of *H. capsulatum* fractions.

V, VI, and VIII by lower optical density and by having two maxima, one at 235 mμ and the second at 241 mμ and a sharp peak at 272 mμ. The difference in the shape of the curves of Fractions V and VI is quite obvious from Fig. 2.

The difference between Fractions V and VIII is not so prominent. Fraction V has lower optical density at 220 mμ than Fraction VIII and higher at 320 mμ. Its small maxima are in different places as compared to Fraction VIII. These differences may be interpreted as significant.

Discussion

The present study was directed towards the determination of the antigenic structure of *H. capsulatum*. The data obtained show the complexity of antigenic organization of this organism. At least seven and perhaps eight antigenically distinct fractions were obtained which could be distinguished with the aid of serology, chemical analysis, and ultraviolet absorption spectra. It is regrettable that it was impossible to prepare antisera against each of these fractions, which would have permitted the demonstration of their antigenic differences more clearly. However, sufficient evidence was obtained to demonstrate with certainty that at least some of these fractions are antigenically different from each other and sufficient indication was obtained in the case of others to assume that they are also antigenically distinct. Thus the results in Table III clearly show that Fraction I is antigenically different from the others. The reactivity of Fraction II (Table IV) with *Blastomyces* antiserum, but not with No. 4 or No. 5 coccidioidal antisera antigenically differentiates it from the other fractions. In the same table conclusive proof is presented showing that Fraction III antigenically differs from the rest and is group specific. No definite serological proof was obtained to indicate that Fraction IV is a distinct antigen; however, evidence is presented in Table II (3rd-week bleeding) indicating that serologically it differs from all the fractions except Fractions VI and VIII. However, it is differentiated from Fraction VIII by the results recorded in Table III. Slower antibody response to Fraction IV (Table II, 5th-week bleeding) as compared to Fraction VI may be interpreted as an evidence of it being antigenically different from Fraction VI. Difference in the ultraviolet absorption spectra of these two fractions (Fig. 2) supports this assumption. But since none of the fractions were sufficiently pure the latter evidence should be taken with reservation. The results recorded in Tables II and IV when analyzed jointly show that Fraction V is distinct from the rest of the fractions. This assumption is based on the fact that serum of 2nd-week bleeding (Table II) reacted with Fractions V and IX whereas coccidioidal serum No. 5 (Table IV) reacted with Fraction V but not with Fraction IX, thus differentiating the two. The evidence presented here showing that antibodies for various fraction appear in infected rabbit at various times during the course of illness may have some bearing on the serodiagnosis of histoplasmosis.

The results reported earlier show that this also holds true with human sera (5). Experiments which are in progress now and which will be reported separately confirm our earlier observations that antibodies for various fractions

appear at different times during the course of illness. This fact obviously is of considerable importance from the standpoint of diagnosis and must be borne in mind in selecting diagnostic antigens. This fact may also account for the conflicting results reported in the literature regarding the reactivity of different antigens.

Summary

1. Procedures of fractionation of yeast phase *H. capsulatum* are outlined.
2. Evidence is presented to indicate that eight isolated fractions are antigenically distinct.
3. Some evidence is presented that antibodies for various fractions appear at different times.
4. It is suggested that in choosing the diagnostic serological antigen the latter statement must be borne in mind.

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THE USE OF AGARASE FROM PSEUDOMONAS ATLANTICA IN THE IDENTIFICATION OF AGAR IN MARINE ALGAE (RHODOPHYCEAE)[1]

W. YAPHE

Abstract

An extracellular hydrolase, specific for agar, was prepared from *Pseudomonas atlantica*. This enzyme hydrolyzed commercial American Difco agar, New Zealand Davis agar, and extracts from *Gelidium cartilagineum*, *G. coulteri*, *G. pristoides*, *Pterocladia pyramidale*, *Gracilaria confervoides*, and *Suhria vittata*. Extracts from algae which contain carrageenin as structural polysaccharide were not hydrolyzed. The products of enzymic hydrolysis were sugars with $R_{galactose}$ of 1.35, 0.75, 0.32, 0.12, 0.07. These sugars constituted a homologous series of oligosaccharides of which neoagarobiose was the basic unit.

Introduction

Agar-decomposing bacteria were first isolated by Gran (6). Humm (7) has reviewed the literature on this subject. The preparation of agarase from marine bacteria has been investigated by Ishimatsu, Kibesaki, and Minamii (8), and the optimum conditions of hydrolysis determined. Araki (1) has shown that a disaccharide agarobiose is formed on acid hydrolysis and an isomer neoagarobiose (3) in enzymic hydrolysis of commercial Japanese agar. Both disaccharides are composed of D-galactose and 3,6-anhydro-L-galactose (2).

The word "agar" is used to describe a preparation containing a specific polysaccharide and is also used to characterize other gel forming substances which are suitable for bacteriological purposes. The polysaccharide agar is obtainable by extraction with hot water from several species of red algae which have in consequence been designated by Tseng (13) as agarophytes. Commercial agar in the United States, Pacific coast agar, is prepared mainly from *Gelidium cartilagineum*; in South Africa and Australia from *Gracilaria confervoides*; in New Zealand from *Pterocladia* spp.; in U.S.S.R. from *Ahnfeldtia plicata* and in Japan mainly from *Gelidium amansii*, but it may also contain extracts from other red algae. The chemical identity of these various preparations remains to be established. Some confusion exists since commercial preparations designated as agar (9) contain the polysaccharide carrageenin. Carrageenin occurs in the red algae which have been designated as carrageens (13). British agar which was used as a substitute for agar was prepared from the carrageens *Chondrus crispus* and *Gigartina stellata*. The products such as Danagar, which is prepared from *Furcellaria fastigiata* and *Hypnea* agar (Atlantic coast agar U.S.A.) prepared from *Hypnea musciformis* contain polysaccharides which are similar to the kappa fraction of carrageenin (14).

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The object of the present study was to use an agarase as a means of distinguishing the presence of agar in marine algae and to obtain evidence for a critical definition of agar.

Materials and Methods

Organism

Agar-decomposing bacteria were isolated from sea water and marine algae in the neighborhood of Halifax, N.S. A strain of *Pseudomonas atlantica* (7) which actively hydrolyzed agar was used for the preparation of the agarase. It was isolated from a specimen of *Rhodymenia palmata* collected at Point Pleasant Park, Halifax, N.S., in November 1952. The organism had the following characteristics: rods 0.5 to 3.0 μ by 0.5 μ occurring singly and in pairs, motile with polar flagella, nonsporeforming, nonencapsulated, Gram-negative. Agar colonies: white, punctiform, smooth, entire, raised, translucent, forming a wide deep depression on agar. Broth: flocculent growth, moderate clouding. Gelatin stab: liquefied stratiform. Indole not formed. Nitrite not produced from nitrate. Hydrogen sulphide produced. Acid from glucose, galactose, lactose, sucrose, maltose, trehalose, dextrin, glycogen. No acid from fructose. Sugars not utilized: arabinose, xylose, cellobiose, raffinose, inulin, glycerol, adonitol, mannitol, sorbitol, dulcitol, salicin, alpha methyl glucoside. Starch, hydrolyzed. Celullose not hydrolyzed. No depression formed by colonies on surface of carrageenin gel. Aerobic: growth at 10° and 37° C.

Growth Medium and Preparation of Agarase

The enzyme was prepared by growing *P. atlantica* in a medium containing per liter of distilled water: NaCl, 25 g.; K_2HPO_4, 0.1 g.; $MgSO_4.7H_2O$, 5.0 g.; $CaCl_2$, 0.2 g.; disodium salt of ethylene diamine tetraacetate (Versene), 0.5 g.; $FeSO_4.7H_2O$, 0.02 g.; KCl, 1.0 g; Casamino acids, 1.0 g.; tris(hydroxymethyl)aminomethane (Tris), 0.4 g.; Difco agar, 3.0 g. The pH of the solution after autoclaving was 7.25. After inoculating 500 ml. of the medium with 10 ml. of a 24 hour culture of *P. altantica*, the flask was placed on a rotary shaker for 3 days at 20° C. The solution was clarified by passing through a Sharples supercentrifuge. The Sharples effluent was filtered through a Selas No. 03 porcelain filter. To the clear Sharples effluent at 3° C., one and two volumes of ethanol were added successively and the precipitates were removed and discarded. The crude enzyme was precipitated by increasing the concentration of ethanol to three volumes.

Source of Algae

Specimens of *Chondrus crispus*, *Gigartina stellata*, and *Halosaccion ramentaceum* were collected at Herring Cove, N.S. *Gelidium cartilagineum* was obtained from Difco Laboratories Inc. Sea Plant Chemical Corporation supplied samples of *Gracilaria confervoides*, *Gigartina acicularis*, *G. pistillata*, *Iridophycus capensis*, *Iridophycus* spp., *Hypnea musciformis*, *Yatabella* spp., Danagar, the extract from *Furcellaria fastigiata*, and commercial New Zealand (Davis) agar, the extract from *Pterocladia* spp. The Institute of Seaweed

Research, Inveresk, provided air-dry ground samples of *Gelidium pristoides*, *G. coulteri*, *Pterocladia pyramidale*, *Suhria vittata*, *Gigartina stiriata*, *G. radula*, *G. christata*, *Rhodoglossum affine*, *Endocladia muricata*, *Ceramium rubrum*, *Dilsea edulis*, *Agardhiella tenera*. The latter algae were analyzed by Ross (1953) for ash and sugars in acid hydrolyzates (11).

Preparation of Algal Extracts

Extracts were prepared from various species of marine algae as follows. Five grams of the dried alga were extracted with 100 ml. of a 0.1% NaCl, 0.1% Versene, 0.1% Tris buffer solution dissolved in distilled water. The solution had a pH 7.2. The alga was first extracted for 15 minutes at 70° C. The mixture was centrifuged and the supernatant was precipitated with three volumes of ethanol. The residue was re-extracted with 100 ml. of buffer in an autoclave for 15 minutes at 120° C. The extract was precipitated in three volumes of ethanol. The extracts at 70° C. and 120° C. were dehydrated successively with absolute alcohol, acetone, and ether and dried *in vacuo* over P_2O_5.

Preparation of Washed Agar

A pad of glass wool was placed on the bottom of a chromatography column 35 × 8 cm. Seventy-five grams of agar as a slurry in distilled water was added to the column. The agar was washed continuously with 0.1% Versene adjusted to pH 7.5, until the effluent was free of carbohydrate. Anthrone reagent was used to test for carbohydrate in the effluent. The first 500 ml. of effluent had a yellow color. The agar was washed with 30 liters of Versene followed by 5 liters of distilled water. The washed agar was extruded from the column and dissolved in water at 100° C. The solution was added to three volumes of ethanol and the precipitate was dehydrated successively with absolute ethanol, acetone, and ether.

Method of Hydrolysis

The rate of hydrolysis of agar was followed by measuring the increase in reducing power expressed as galactose (12). One volume of the enzyme was added to two volumes of a 1.5% solution of agar in $M/20$ phosphate buffer. Aliquots were removed at intervals for analyses. Activity was expressed as the amount of galactose formed after hydrolysis of a 1.0% agar solution at 40° C. and pH 6.0 for 30 minutes.

Chromatography

Products of hydrolysis were separated by one-dimensional descending chromatography on Whatman No. 1 filter paper for 24 or 72 to 96 hours. The best separation of the oligosaccharides was obtained with *n*-butanol, pyridine, and water (2:1:1). Spray reagents used to identify the sugars on the chromatogram were aniline hydrogen phthalate and a modified Seliwanoff reagent. The latter contained one volume of 0.2% napthoresorcinol and one volume of an ethanol sulphuric acid solution (375 ml. of ethanol plus 100 ml. of concentrated sulphuric acid). After spraying with the Seliwanoff

reagent the chromatograms were left at 20° to 25° C. Galactose, glucose, and mannose gave a blue spot in 30 to 45 minutes; fructose and 5-hydroxy-methyl-2-furaldehyde (H.M.F.) a red spot in 2 minutes; 3,6-anhydrogalactose a blue spot in 15 minutes. This reagent was more sensitive than aniline hydrogen phthalate to low concentrations of 3,6-anhydrogalactose and neoagarobiose.

Results

Effect of pH

The effect of pH on enzymic activity is shown in Table I. The enzyme was active over a pH range of 5 to 8 with optimum activity about 6.0.

TABLE I

THE RELATIONSHIP BETWEEN pH AND ENZYMIC ACTIVITY. REDUCING SUGAR FORMED ON HYDROLYSIS OF 1.0% SOLUTION OF AGAR AT 40° C. FOR 30 MINUTES. ENZYME STERILE SHARPLES EFFLUENT

pH	4.9	5.35	5.75	6.25	6.80	7.40	7.70
Mg. of gal./100 ml.	45.6	58.8	68.1	66.0	64.0	60.0	40.5

Activity Curve

The rate of hydrolysis of agar by the sterile Sharples effluent and the concentrated agarase at pH 6, 40° C. is shown in Fig. 1.

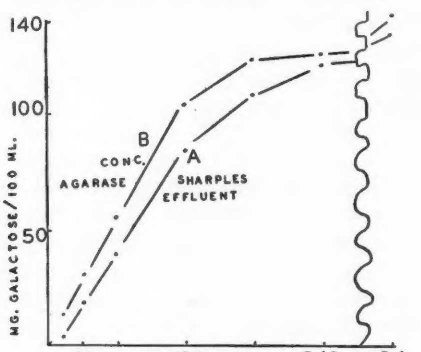


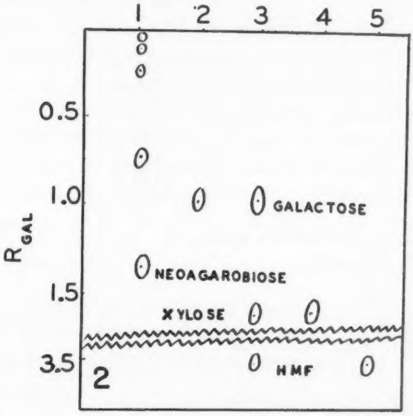
FIG. 1. Time activity curve. Rate of hydrolysis of 1.0% agar at 40° C., pH6.0. Enzyme A, sterile Sharples effluent; Enzyme B, ethanol-concentrated agarase.

Effect of Temperature

The minimum temperature for the assay of agarase activity is 40° C. since the mixture gels below 40° C. The enzyme was rapidly inactivated above 50° C. Sterile preparations of the enzyme which were stored at 3° C. showed no loss of activity.

Effect of Substrate and Enzyme Concentration

Variation in the concentration of substrate showed that enzymic activity was independent of concentration above 0.3% agar. Variation in enzyme concentration showed that enzymic activity was proportional to enzyme concentration.



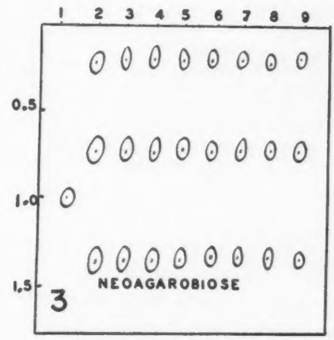


FIG. 2. Chromatogram of enzyme and acid hydrolyzates of agar. 1, enzyme hydrolzate; 2, galactose; 3, acid hydrolyzate; 4, xylose; 5, hydroxymethyl-2-furaldehyde (H M.F.). $R_{gal.}$ is the movement of the sugars relative to that of galactose.

FIG. 3. Chromatogram of enzymic hydrolyzates of extracts from marine algae. 1, galactose; 2, *Gelidium cartilagineum*; 3, *G. coulteri*; 4, *G. pristoides;* 5, *Pterocladia pyramidale*; 6, *Gracilaria confervoides*; 7, *Suhria vittata*; 8, washed Difco agar; 9, Davis agar. $R_{gal.}$ is the movement of the sugars relative to that of galactose.

Products of Hydrolysis

The enzymic hydrolyzate of washed commercial agar (Difco lot No. 430790) was chromatographed. Sugars were obtained with $R_{gal.}$ values of 1.35, 0.75, 0.32, 0.12, 0.07 (Fig. 2). Araki (3) showed that neoagarobiose had an $R_{gal.}$ of 1.30. The plot of log $(1/R_f - 1)$ versus disaccharide units was a straight line. This indicated a homologous series of oligosaccharides with the disaccharide neoagarobiose as the first sugar (5). The washed agar was heated for 12 hours in 0.1 N H_2SO_4 in a sealed tube at 100° C. The hydrolyzate was neutralized with $BaCO_3$, filtered, and chromatographed. Spots were obtained with R_f values identical with those of galactose, xylose, and H.M.F. (Fig. 2). The spot for xylose indicated that the commercial agar probably contained a xylan. The H.M.F. was formed from 3,6-anhydro-L-galactose (10).

Hydrolysis of Algal Extracts

The extracts from marine algae and the commercial agars were incubated with the agarase at pH 6.0 and 40° C. An increase in reducing sugar was obtained with extracts from the algae in Table II. The reducing power of the extracts varied from 45.6 mg., for material extracted from *Gelidium cartilagineum* at 70° C. to 150 mg., for the washed Difco agar. Chromatograms were

TABLE II

ENZYMIC HYDROLYSIS OF EXTRACTS FROM MARINE ALGAE. REDUCING SUGAR FORMED ON HYDROLYSIS OF 1.0% SOLUTION OF EXTRACT AT 40° C., pH 6.0, for 24 HR. ENZYME STERILE SHARPLES EFFLUENT

Extract		Reducing sugar, mg. 100 ml.
Gelidium cartilagineum	E-70°	45.6
G. cartilagineum	E-120°	137.0
G. pristoides	E-120°	119.1
G. coulteri	E-120°	102.0
Gracilaria confervoides	E-70°	86.7
Pterocladia pyramidale	E-120°	93 0
Suhria vittata	E-120°	108.6
Washed Difco agar (U.S.A.)		150.0
Davis agar (New Zealand)		129.3
Control extracts plus heated enzyme		0

E-70, E-120, algae extracted successively at 70° and 120° (see text)

run with the enzymic hydrolyzates of extracts from algae in Table II. Spots were obtained for sugars with identical $R_{gal.}$ values of 1.35, 0.72, 0.30 (Fig. 3). The algal extracts were also hydrolyzed with 0.1 N H_2SO_4 as described above. The neutralized hydrolyzates were chromatographed and sugars with the same $R_{gal.}$ values as galactose, xylose, and H.M.F. were obtained. The enzyme preparation was specific for agar. No increase in reducing sugar was obtained with extracts from the following red algae: *Chondrus crispus, Gigartina stellata, G. stiriata, G. acicularis, G. radula, G. pistillata, G. christata, Iridophycus* sp., *Iridophycus capensis, Rhodoglossum affine, Hypnea musciformis, Furcellaria fastigiata, Yatabella* sp., *Endocladia muricata, Ceramium rubrum, Dilsea edulis, Agardhiella tenera, Halosaccion ramentaceum.*

Discussion

Although agar has been used by bacteriologists since 1882, no adequate standards of purity or origin of the commercial product have been defined. From recent chemical investigations agar may be defined as a polysaccharide made up exclusively of disaccharide repeating units of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose (2, 4, 10). This polysaccharide may be further defined on the basis of gel strength, viscosity, and ash content. A definition for agar should include the name of the alga from which it was prepared. This communication draws attention to the many possible sources of agar. Evidence is presented to show that agar is the structural polysaccharide in *Gelidium cartilagineum, G. pristoides, G. coulteri, Gracilaria confervoides, Pterocladia pyramidale, Pterocladia* spp. (Davis agar), *Suhria vittata.* The products of hydrolysis by agarase of extracts from these agarophytes consist of a homologous series of oligosaccharides with identical $R_{gal.}$ values. A reducing sugar of $R_{gal.}$ 1.35 was obtained which is in reasonable agreement with the $R_{gal.}$ of 1.30 for neoagarobiose. Acid hydrolyzates of the extracts contained galactose and H.M.F. Under the conditions of

hydrolysis used the H.M.F. would not be formed from galactose but would be formed from 3,6-anhydrogalactose (10). This may be taken to indicate that the extracts contained polysaccharides which were hydrolyzed by an enzyme specific for agar and that the basic sugar units of these polysaccharides were galactose and 3,6-anhydro-L-galactose.

Commercial agar prepared from different algae varies in gel strength. Araki (2) proposed the terms agarose and agaropectin for the gelatinous and nongelatinous constituents in Japanese agar. The amount of reducing sugar formed on enzymic hydrolysis of extracts from the agarophytes is indicative of the amount of the gelatinous agarose in the extract.

Since the structure of agar is known it is proposed (*a*) that the word "agar" should only be used to describe preparations which contain this polysaccharide; (*b*) that information is now available to establish standards for bacteriological agar.

Acknowledgment

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SURVIVAL OF PSEUDOMONAS AERUGINOSA (SCHROETER) MIGULA SUSPENDED IN VARIOUS SOLUTIONS AND DRIED IN AIR[1]

JUNE M. STEPHENS[2]

Abstract

When added to aqueous suspensions of *Pseudomonas aeruginosa* (Schroeter) Migula, an organism pathogenic to grasshoppers in the laboratory, a number of test agents allowed no survival after 24 hours' desiccation in air; others, 0.001 to 10%; 5% sucrose, 30%. A combination of 1% casein, 5% sucrose, and 1% granular mucin supported the growth of the bacteria and gave 29% survival after 24 hours' drying. Survival rates in this investigation compare favorably to those obtained by other investigators when drying was carried out under complex conditions, such as freeze-drying.

Introduction

Pseudomonas aeruginosa (Schroeter) Migula, a bacterium pathogenic to grasshoppers in the laboratory (2), shows little resistance to desiccation (2, 5). This investigation was made to find an agent that would increase the resistance of the bacteria to the desiccation to which they would be exposed during field trials against grasshoppers.

Most investigations on the survival of bacteria after desiccation have been conducted for storing of stock cultures. The desiccation usually has been carried out *in vacuo* at low temperatures and investigators have been more concerned with the choice of a suspending medium for freeze-drying than with measurement of percentage survival during drying and of time during which greatest mortality occurs. In this investigation, percentage survival was recorded during the drying period. Proom and Hemmons (6) reported that the percentage of bacteria that survived the freeze-drying process varied from 100% to less than 1%, depending on the species. Though their results are not quantitative, they referred to a species of *Pseudomonas* dried at −78° C. that did not survive as well as did many of the other species. Stamp (8), in a study of suspending media for several species of bacteria, found gelatin the most satisfactory medium. He gave a quantitative record of survival immediately after drying and found that some species showed less than 1% survival in 2 to 3 days after freeze-drying.

Most of the adjuvants used with insect pathogens have been used to stick organisms to plants or foliage rather than to aid in their survival (1, 3, 9). The primary aim in formulating bacterial sprays for insect control should be to secure a formula that will allow a high percentage of organisms to survive in the deposit for a short period, e.g., about a week, during which the insect population may be expected to become infected. Few investigators have reported on the survival of organisms under these conditions. Morton and

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Pulaski (5) reported that slow desiccation in air appears to have a highly lethal effect on bacteria and that alternating conditions of humidity and dryness are even more destructive. For the purpose of the present investigation drying was carried out in air under more or less constant conditions of temperature and humidity.

The substances tested included those that were found valuable in freeze-drying procedures and others that are considered to prevent complete desiccation of a film. There were four criteria for choosing a test substance: (1) it must be a compound that has a sticking or coating quality; (2) it must be a compound that in desired concentration is suitable for spraying; (3) it must be nontoxic to the bacteria; and (4) it must be inexpensive so as to be practical for field use.

Materials and Methods

Culture

A strain of *P. aeruginosa* isolated from grasshoppers and designated as P11-1 (2) was used in all experiments.

Preparation of Suspending Menstruum

All substances were prepared in aqueous solution and the pH was adjusted with $N/10$ NaOH to 7.4, the optimal pH of the test strain (2). Whenever possible the material was sterilized at 15 lb. pressure for 15 minutes. Concentrations of the test substances ranged from 0.1% to 10%, depending on the coagulating properties of each. At high concentrations many of the test substances coagulated and became unsuitable for spraying.

Preparation and Addition of Bacterial Suspension

Cultures were grown in Bacto-nutrient broth (Difco) for 20 to 24 hours at 37° C. and centrifuged, and the packed cells washed with sterile water. Twenty-hour cultures of paracolon bacilli were considered more resistant than those of other ages when dried *in vacuo* from the frozen state (4). Cultures of P11-1 older than 24 hours were not used as every effort was made to maintain bacteria in the most virulent state. Sufficient cells were added to 100 ml. of the test solution to give a final concentration of about 10^9 bacteria per ml.

The suspensions were stored for 7 days at room temperature, when a viable count was made by the "drop plate" method of Reed and Reed (7) to determine whether the protective substance in aqueous solution had any deleterious effect on the bacteria. The suspension was considered suitable to be tested for its protective action if no significant decrease in number had occurred after 7 days.

Drying of Bacteria in Test Substances

One drop of the suspension to be tested was delivered from a calibrated pipette to a sterile cover slip (18 mm. circle). One drop from the calibrated pipette was equivalent to 1/40 ml. A sufficient number of cover slips were prepared to allow daily samples for 2 weeks if necessary.

Drops of test solution were allowed to dry on cover slips at a constant temperature of 30° C. and a relative humidity of 10 to 20%. Desiccated droplets were maintained at this temperature and humidity throughout the experimental period. At time of sampling a cover slip was crushed in a dilution tube by grinding with a sterile glass rod until the fine glass particles could be evenly suspended by shaking.

Organisms in test solutions were also dried under the same conditions on 10-mm. circles of hardened parawax on sterile glazed paper. Counts on cover slips and wax droplets were not significantly different for 18 replicates so all results are expressed as survivals on cover slips.

Estimation of Survival

Counts of the viable bacteria per drop were made immediately after visible drying (in about 0.5 to 1 hour) and at daily intervals until results were consistently negative. The number of surviving organisms at the time of sampling was calculated as a percentage of the number in the original drop immediately after visible drying. There was little trouble with contamination as aseptic conditions were maintained during the experiment. Only those substances that showed at least 1% survival after 2 days were considered to offer a sufficient degree of protection to warrant further tests.

Results

Agents That Offered No Protection

No survival of *P. aeruginosa* was detected with the following agents: 0.25, 0.5, and 1% ascorbic acid; 1, 2, and 3% cornstarch; 0.05 and 0.1% cystine; 0.05 and 0.1% egg albumin; 3% Florosil, 3% Florigel, and 3% Diluex A (Floridin Company, Warren, Penn.); 0.5, 1, and 5% glycerol; 1% Polyglycol E 400 and 5% Polyglycol E 400, 1% Polyglycol P 400 and 5% Polyglycol P 400 (Dow Chemical of Canada Limited); 0.1% molar mixture of sodium chloride and calcium chloride; 1% sodium oleate; 1, 2, and 5% Sorbo (Atlas Powder Company of Canada, Limited); 1% tryptone; and 0.25 and 0.5% thiourea.

Agents That Offered Limited Protection

All substances that offered any protection to *P. aeruginosa* are listed in Table I. In most cases where protection was slight an increase in concentration of the suspending agent had no effect on the percentage survival. Survival was independent of the original concentration of *P. aeruginosa* cells: increasing the concentration from 10^9 to 10^{12} cells per ml. did not increase the percentage survival. Therefore all survivals listed are for a concentration of approximately 10^9 cells per ml.

Contrary to the findings of Stamp (8), gelatin was not a good suspending medium. No better results were obtained when it was tried in combination with ascorbic acid, which Stamp (8) also found to have a favorable effect.

TABLE I

PERCENTAGE SURVIVALS AT VARIOUS INTERVALS OF P11-1 STRAIN OF *P. aeruginosa* SUSPENDED IN AQUEOUS SOLUTIONS OF VARIOUS AGENTS AND DRIED ON COVER SLIPS

(Average of duplicate experiments)

Suspending agent	% concentration	% survival 1 day	2 days	4–7 days	10–14 days
Agar (Bacto)*	0.05	0.001	0	0	0
Casein	0.05	1	1	0.2	0.01
	0.1	6	5	2	0.4
	0.5	10	1	1	0.1
	1	10	3	1	0.1
Corn steep	5	10	0	0	0
Defibrinated rabbit blood	1	0.3	0.01	0.002	0
Dextrin	0.5	0.03	0	0	0
Gelatin	0.25	0.4	0.4	0	0
	0.5	3	0	0	0
Glucose	1	3	0.02	0.005	0
	5	3	0.02	0.01	0
Gum arabic	0.05	0.02	0	0	0
	0.1	0.02	0	0	0
Methocel†	1	0.04	0.005	0	0
	5	0.04	0.004	0	0
Skim milk (Bacto)*	0.5	5	0.5	0.001	0
Mucin (gastric)‡	1	0.8	0.08	0.07	0
Mucin (granular) Type 1701-W‡	1	2	1	0.1	0
Nutrient broth (Bacto)*		0.5	0.003	0	0
Peat (powdered)	1	0.004	0	0	0
Peptone (Bacto)*	1	0.2	0	0	0
Rubber latex§	0.25	0.02	0	0	0
Sucrose	1	5	3	3	1
	5	30	4	2	0.2
	10	25	5	1	0.08
	15	29	4	2	0.04
Water		0.02	0	0	0

*Difco Laboratories, Incorporated, Detroit, Michigan.
†Dow Chemical of Canada, Limited.
‡Wilson Laboratories, Chicago, Illinois.
§Goodyear Tire and Rubber Company, Akron, Ohio.

TABLE II

PERCENTAGE SURVIVALS AT VARIOUS INTERVALS OF P11-1 STRAIN OF *P. aeruginosa* IN DIFFERENT MIXTURES OF CASEIN, SUCROSE, AND GRANULAR MUCIN AND DRIED ON COVER SLIPS

(Average of duplicate experiments)

Mixture	% survival 1 day	2 days	4–7 days	10–14 days
1% casein, 1% sucrose, and 1% granular mucin	13	6	6	1
5% casein, 5% sucrose, and 5% granular mucin	20	6	3	1
1% casein, 5% sucrose, and 1% granular mucin	29	13	9	2

In this group of substances, only casein, granular mucin, and sucrose offered as much as 1% protection after 2 days' drying. The use of Tween 80 (Atlas Powder Company of Canada, Limited) as a wetting agent with these substances did not yield better results.

Survival in Casein, Sucrose, and Granular Mucin

Individual results with casein, sucrose, and granular mucin indicated that sucrose probably had the greatest effect of these three. Table II shows that 5% concentrations of casein and granular mucin in combination with 5% sucrose do not give as good survival as when they are used in 1% concentration with 5% sucrose.

The duration of storage of organisms in the mixture of casein, sucrose, and granular mucin before application to cover slips did not have a noticeable effect upon the percentage survival after desiccation. Organisms stored for up to 28 days before drying showed survivals comparable to those stored for only 7 days.

It is noteworthy that a mixture of 1% casein, 5% sucrose, and 1% granular mucin supported the growth of *P. aeruginosa.* This feature added to the value of the mixture as a suspending agent for the bacteria in field trials as much of the time-consuming manipulation of centrifuging and resuspending cells could be eliminated. Percentage survival of bacteria grown in the mixture was about equal to that obtained when cells were grown in nutrient broth before they were added to the mixture.

Discussion

Cells of *P. aeruginosa* are very susceptible to desiccation even when protected by a mixture of casein, sucrose, and granular mucin, the most effective agent in the group tested. Though there are no previous reports of the survival of *P. aeruginosa* dried under the conditions used in this investigation, the species falls into the group of nonresistant bacteria when stored in sealed ampoules (5).

Fry and Greaves (4) found that very rapid drying in air of a strain of paracolon bacillus suspended in 7.5% glucose was about as lethal as the slower process of freeze-drying. Survivals of *P. aeruginosa* after 1 week's drying ranged from less than 1%, in a variety of substances, to as much as 9%, in a mixture of casein, sucrose, and granular mucin. Though these figures are not phenomenal, they compare favorably to 3–9 day survivals of the paracolon bacillus dried *in vacuo* from the frozen state at high temperatures (4). One striking difference is that gelatin and glucose were the best suspending media for the paracolon bacillus though they are of little value for *P. aeruginosa.*

This investigation showed that sucrose offered the best protection to *P. aeruginosa* at a concentration of about 5%. The mean survival of the paracolon bacillus in 5 to 10% glucose, its most effective suspending medium, after 24 hours' freeze-drying was reported by Fry and Greaves (4) to be 32%. Survivals with *P. aeruginosa* in sucrose or a mixture of casein, sucrose, and

granular mucin were 20 to 30% for the same period. Fry and Greaves found that sucrose gave a steady increase in survival with increasing concentrations up to 50%. With *P. aeruginosa*, the effect of sucrose appears to level off in the 5 to 10% range. Fry and Greaves (4) stated that it is difficult to get strong solutions of sucrose even approximately dry and this fact may possibly contribute to the protective action of sucrose for *P. aeruginosa*.

The reasons why one agent offers protection though another of similar chemical properties has no effect are not clear. For example, sucrose offers some protection whereas glucose does not. There is very little possibility that any of the suspending agents were toxic to the bacteria as all the aqueous suspensions of test mixtures survived equally well.

No attempt was made in this investigation to determine the mortality rate of bacteria during the first 24 hours of drying. As the number of organisms per drop immediately after visible drying was not greatly different from the number applied, scant decrease in number occurred within the first hour after application. It appears therefore that insects must consume bacteria very early after their application in the field if maximum numbers are to be ingested. As there is no assurance as to the time when insects will feed on sprayed foliage, they would probably consume food infected with less than 20% of the original number of bacteria applied.

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THE ASPARAGINE DEAMIDASE OF BACILLUS COAGULANS AND BACILLUS STEAROTHERMOPHILUS[1]

Gilbert B. Manning and L. Leon Campbell, Jr.

Abstract

The purification and certain properties of L-asparagine deamidase of *Bacillus coagulans* and *Bacillus stearothermophilus* are described. Maximum enzyme activity was obtained at 55° C. over a pH range of 7.5 to 8.5. The purified deamidase hydrolyzed the L-isomer of asparagine quantitatively to aspartic acid and ammonia and did not attack the D-isomer. D-Asparagine inhibited the hydrolysis of the L-isomer. The K_m for the inhibition reaction was found to be 1.87×10^{-2} *M*. The enzyme did not catalyze transamidation or hydroxylamine transfer reactions. Enzyme activity was inhibited by N-ethylmaleimide and *p*-chloromercuribenzoate. The inhibition by these agents was reversed by glutathione. In the absence of substrate the deamidase of both organisms was relatively heat labile at 55° C. The heat lability of this enzyme is discussed in relation to the heat stability of other enzyme systems of thermophilic microorganisms.

Introduction

Asparagine can be deamidated by any one of the following enzymatic reactions: (*a*) hydrolysis to aspartic acid and ammonia, which may or may not be phosphate dependent, (*b*) deamidation which requires the presence of an α-keto acid, and (*c*) deamidation associated with ω-replacement reactions (6, 16, 17, 27). Altenbern and Housewright (2) reported the presence of two asparagine deamidases in sonic extracts of *Brucella abortus*, one specifically hydrolyzing the L-isomer and one hydrolyzing the D-isomer of asparagine.

The present report is concerned with the partial purification and properties of the asparagine deamidase of *Bacillus coagulans* and *Bacillus stearothermophilus*. The purified deamidase from both organisms is specific for the L-isomer of asparagine, hydrolyzing it to aspartic acid and ammonia.

Materials and Methods

Organisms

The facultative thermophile *Bacillus coagulans* strain 43P and the obligate thermophile *Bacillus stearothermophilus* strain 1503 were selected for study. Stock cultures were maintained by monthly transfer on Difco nutrient agar slants.

Materials

D- and L-Asparagine and the amino acids tested were obtained from the Nutritional Biochemicals Corp. N-Ethylmaleimide and *p*-chloromercuribenzoate were purchased from the Mann Research Laboratories. Formamide, acetamide, propanamide, *n*-butyramide, isobutyramide, valeramide, hexan-

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amide, acrylamide, oxamide, succinimide, and salicylamide were obtained from the Eastman Kodak Company. Calcium phosphate gel was prepared according to Strittmatter and Ball (26).

Methods

Ammonia was determined by nesslerization employing the procedure of Koch and McMeekin (9). Color development was measured at either 480 or 540 mμ using a Bausch and Lomb "Spectronic 20" colorimeter. Protein was determined by the method of Lowry *et al.* (12). Aspartic acid was determined manometrically, using dried cells of *Clostridium perfringens* (ATCC 8009) as described by Meister *et al.* (18). Aspartic acid was identified by descending paper chromatography on Whatman No. 1 paper with phenol–water (80:20) as the solvent. Papers were dried and developed with ninhydrin by conventional procedures.

Enzyme Assay

Asparagine deamidase activity was measured routinely in reaction mixtures containing 1.0 ml. of enzyme, 50 μM. L-asparagine, and 0.05 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.3 in a total volume of 4.0 ml. After 1 hour incubation at 55° C. the reaction was stopped by the addition of 0.1 ml. of 3.0 *M* trichloroacetic acid. The precipitated protein was removed by centrifugation and the supernatant liquid analyzed for ammonia. One unit of enzyme is defined as that amount of protein which forms 1.0 μM. ammonia from L-asparagine per hour at 55° C. Specific activity is expressed as micromoles of ammonia formed per milligram protein per hour.

Enzyme Production

The medium used for enzyme production had the following composition: tryptone (Difco) 20.0 g.; yeast extract (Difco) 5.0 g.; salts B (25) 1.0 ml.; distilled water to 1 l., pH 7.2. To prevent excessive foaming during aeration, Dow Corning antifoam A was sprayed over the surface of the medium prior to sterilization.

Carboys containing 15 l. of the tryptone–yeast extract medium were inoculated with 500 ml. of an 18 hour broth culture of the organism under study. The carboys were then incubated in a 55° C. water bath for 36 hours with constant aeration (500 ml. of sterile air per minute). The cells were harvested by centrifugation with a Sharples super speed centrifuge, washed three times with 0.01 *M* potassium phosphate buffer, pH 8.3, and lyophilized on a Machlett macro freeze-dry apparatus. The dried cells were stored at −10° C. until used for enzyme extraction.

Purification of Asparagine Deamidase

(*a*) ***Crude Cell-free Extract***

Five grams of dried cells were ground for 10 minutes in a mortar at room temperature. The fine powder was suspended in 100 ml. of 0.05 *M* Tris buffer, pH 8.6, and incubated for 1 hour at 37° C. with intermittent shaking. The cell debris was removed by centrifugation at 25,000$\times g$ for 15 minutes at

room temperature, using a Servall superspeed centrifuge. Cell extracts could also be prepared by grinding the dried cells with alumina or by subjecting them to sonic vibration in a Raytheon 9 kc. sonic oscillator. However, owing to the ease of preparation the extracts were routinely prepared as described above. Such extracts were equal in activity to the sonic or alumina extracts.

(*b*) *Sodium Sulphate Fraction*

Sodium sulphate (17.5 g.) was added to 100 ml. of the clear straw-colored supernatant liquid with constant stirring. The precipitate was removed by centrifugation at $25{,}000 \times g$ for 15 minutes at room temperature and discarded. Sodium sulphate (14.0 g.) was added slowly with stirring to the supernatant liquid and the precipitate collected by centrifugation at $25{,}000 \times g$ for 1 hour at room temperature. The precipitate was taken up in 50 ml. of 0.05 *M* Tris buffer, pH 8.6.

(*c*) *Calcium Phosphate Fraction*

To the enzyme solution obtained in (*b*) was added one-half volume of calcium phosphate gel. The gel–enzyme mixture was stirred for 15 minutes and the gel collected by centrifugation. The gel was then washed six times with four volumes of distilled water. Fifty milliliters of 0.1 *M* phosphate buffer, pH 8.3, was added to the gel and the suspension was stirred at room temperature for 15 minutes. The gel was removed by centrifugation and discarded. The supernatant liquid contained the enzyme and was used in the remainder of the study. Table I summarizes a typical purification procedure for the deamidase of *B. coagulans*. Similar purification was obtained with the deamidase of *B. stearothermophilus*. The purified enzyme from both organisms was stable for at least 1 year when stored at −10° C. Repeated attempts to purify the deamidase further by several different fractionation procedures employing ammonium sulphate, acetone, and ethanol, at either room temperature or at 0° to 5° C., met with failure.

TABLE I

PURIFICATION OF ASPARAGINE DEAMIDASE OF *Bacillus coagulans*

Fraction	Units*	Specific activity†
Crude cell extract	524.0	3.94
Na_2SO_4 fraction	386.13	10.55
Phosphate gel eluate	276.78	13.18

*One unit of enzyme is defined as that amount of protein which forms 1.0 μM. of ammonia from asparagine per hour at 55° C.

†Specific activity is defined as micromoles of ammonia released per milligram protein per hour.

Results and Discussion

Deamination of L-*Amino Acids and Amino Acid Amides by Crude Cell Extracts*

Crude cell extracts of *B. coagulans* and *B. stearothermophilus* were capable of deaminating or deamidating L-asparagine, L-glutamine, L-aspartic acid,

L-glutamic acid, L-valine, L-serine, L-threonine, and L-leucine. Arginine, methionine, alanine, cysteine, histidine, and lysine were not deaminated by the crude cell extracts of either organism. The enzyme deamidating L-asparagine was found to be the most active system present in the extracts of both organisms. Typical data are summarized in Table II.

TABLE II

DEAMINATION OF AMINO ACIDS AND DEAMIDATION OF AMINO ACID AMIDES BY CRUDE CELL EXTRACTS OF *Bacillus coagulans* AND *Bacillus stearothermophilus*

Substrate	*B. coagulans*	*B. stearothermophilus*
Aspartic acid	10.8*	18.4
Asparagine	47.8	34.2
Arginine	0	0
Alanine	0	0
Cysteine	0	0
Glutamic acid	12.4	11.2
Glutamine	20.2	15.6
Histidine	0	0
Leucine	9.6	8.4
Lysine	0	0
Methionine	0	0
Threonine	8.0	9.6
Serine	15.6	17.4
Valine	10.8	9.8

*Micromoles ammonia formed under assay conditions. The reaction mixture contained 0.5 ml. cell extract (5.0 mg. protein), 0.5 ml. substrate (50 μM.) and 1.0 ml. of 0.05 *M* Tris buffer, pH 8.3. Reaction was run 1 hour at 55° C.

Effect of pH on Enzyme Activity

The optimum pH range for the deamidation of asparagine was found to be 7.5 to 8.5 (Fig. 1) when run in Robinson's universal buffer. Equal activity was obtained when the reaction was run at pH 8.3 in 0.01 *M* phosphate, 0.05 *M* Tris, or the universal buffer system.

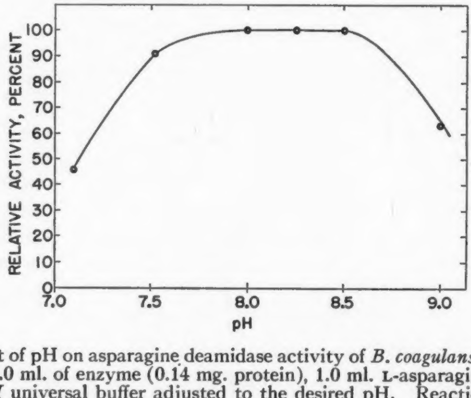


FIG. 1. Effect of pH on asparagine deamidase activity of *B. coagulans*. Reaction mixture contained 1.0 ml. of enzyme (0.14 mg. protein), 1.0 ml. L-asparagine (50 μM.), and 2.0 ml. of 0.2 *M* universal buffer adjusted to the desired pH. Reaction mixtures were incubated for 1 hour at 55° C.

Optimum Temperature for Enzyme Activity

Enzyme activity against asparagine was measured over a temperature range of 35° to 75° C. at increments of 5° C. The deamidase of both organisms had an optimum temperature of 55° C. Above this temperature activity dropped sharply owing to thermal inactivation of the enzyme, as will be shown later.

Effect of Enzyme and Substrate Concentration on Deamidase Activity

The release of ammonia from L-asparagine was found to be linearly related to the enzyme concentration. Fig. 2 shows the data obtained with the deamidase of *B. coagulans*. Fig. 3 shows the effect of substrate concentration on enzyme activity plotted according to Lineweaver and Burk (11). The K_m for the reaction was calculated to be 2.4×10^{-2} M for the deamidase of *B. coagulans* and 8.5×10^{-3} M for the enzyme of *B. stearothermophilus*.

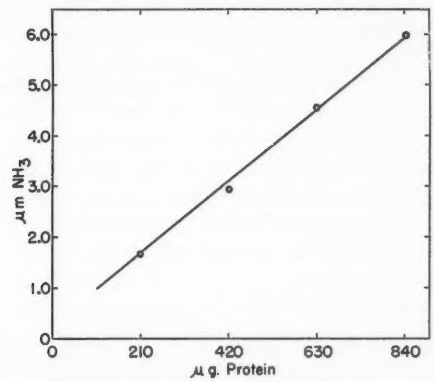


FIG. 2. Effect of enzyme concentration on asparagine deamidase activity of *B. coagulans*. Reaction mixture contained 1.0 ml. of enzyme of the desired protein concentration, 1.0 ml. of L-asparagine (50 μM.), and 2.0 ml. of 0.05 M Tris buffer, pH 8.3. Reaction mixtures were incubated for 1 hour at 55° C.

Balance Studies on Asparagine Hydrolysis

Typical quantitative data on the hydrolysis of L-asparagine are presented in Table III. It can be seen that there is a good agreement between the amount of ammonia released from asparagine and the amount of aspartic acid formed. These data clearly show that the deamidase of both organisms catalyzes the hydrolysis of asparagine to ammonia and aspartic acid.

Specificity of the Purified Deamidase

The purified deamidase of both organisms hydrolyzed the L-isomer of asparagine and did not attack the D-isomer. Grossowicz and Halpern (7) reported that D-asparagine inhibited the deamidation of L-asparagine by crude cell extracts of *Mycobacterium phlei*. This effect was tested with the deamidase of *B. coagulans* by increasing the concentration of the D-isomer in the presence of an optimum amount of L-asparagine. At equal concentra-

TABLE III

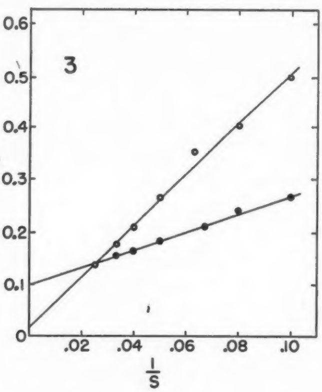
BALANCE DATA FOR L-ASPARAGINE HYDROLYSIS

Deamidase	Products formed	
	Ammonia, μM.	Aspartic acid, μM.
B. coagulans	7.35	7.47
B. stearothermophilus	7.52	7.81

NOTE: Reaction mixtures contained 1.0 ml. of the appropriate enzyme (0.25 mg. protein), 1.0 ml. L-asparagine (10 μM.), and 2.0 ml. of 0.05 *M* Tris buffer, pH 8.3. Incubation was at 55° C. for 1 hour.

tions of the two isomers there was a 31% inhibition of the reaction. Fig. 4 shows a Lineweaver–Burk plot of the data obtained. The half-maximal concentration of D-asparagine for the inhibition of the reaction was calculated to be 1.87×10^{-2} *M*.

Purified deamidase preparations of both organisms were tested for additional activities. The purified enzyme did not deaminate or deamidate any of the L-amino acids or amides (except L-asparagine) that were attacked by the crude cell extracts. Formamide, acetamide, propanamide, *n*-butyramide, isobutyramide, valeramide, hexanamide, acrylamide, oxamide, succinimide, and salicylamide were also not attacked by the purified enzyme. No transaminase or transamidase activity was demonstrated with the deamidase when



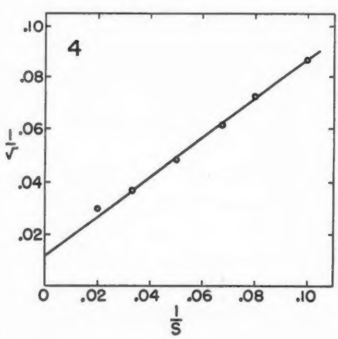


FIG. 3. Lineweaver–Burk plot of asparagine hydrolysis by deamidase preparations of *B. coagulans* (○) and *B. stearothermophilus* (●). Reaction mixtures contained 1.0 ml. of the appropriate enzyme preparation (0.27 mg. protein), 1.0 ml. of L-asparagine of the desired concentration, and 2.0 ml. of 0.05 *M* Tris buffer, pH 8.3. Incubation was at 55° C. for 1 hour.

FIG. 4. Effect of D-asparagine concentration on the inhibition of L-asparagine hydrolysis by deamidase of *B. coagulans*. Reaction mixtures contained 1.0 ml. of enzyme (0.42 mg. protein), 0.5 ml. of L-asparagine (50 μM.), 0.5 ml. of D-asparagine of the desired concentration, and 2.0 ml. of 0.05 *M* Tris buffer, pH 8.3. Incubation was at 55° C. for 2 hours.

L-asparagine was tested with pyruvate, α-ketoglutarate, α-ketobutyrate, or glyoxylate using the methods described by Campbell (5). The deamidase preparations also did not catalyze hydroxylamine transfer reactions when tested according to the procedures of Meister *et al.* (19). These data show that the enzyme preparations studied have a high degree of specificity, catalyzing only the hydrolytic cleavage of L-asparagine to ammonia and aspartic acid.

Effect of N-Ethylmaleimide (NEM) and p-Chloromercuribenzoate (PCMB) on Enzyme Activity

The activity of the deamidase preparations of both organisms was completely inhibited by 1×10^{-4} *M* NEM and by 1×10^{-5} *M* PCMB. With the deamidase of *B. coagulans*, activity was inhibited 31.7% by 1×10^{-5} *M* NEM and 42% by 1×10^{-6} *M* PCMB. Similar data were obtained with the enzyme from *B. stearothermophilus*. Preincubation of the enzyme for 10 minutes at 55° C. with equimolar concentrations of glutathione completely reversed the inhibitory action of both compounds. These data suggest that the enzyme requires free sulphydryl groups for activity.

Thermal Stability of Asparagine Deamidase

To study the thermal stability of the enzyme, samples were placed in a water bath at the desired temperature. At suitable intervals of time, aliquots were removed and the activity tested with L-asparagine under optimum assay conditions. Attempts to study the thermal stability of the enzyme at temperatures of 60° and 70° C. in the absence of substrate met with difficulty owing to the very rapid inactivation of the enzyme at these temperatures. For example, the enzyme from *B. coagulans* was completely inactivated after 5 minutes at 70° C. and showed 95% inactivation after 5 minutes at 60° C. For this reason we studied the thermal stability of the preparations at 55° C. Fig. 5 shows that in the absence of substrate, the enzyme from *B. coagulans* lost

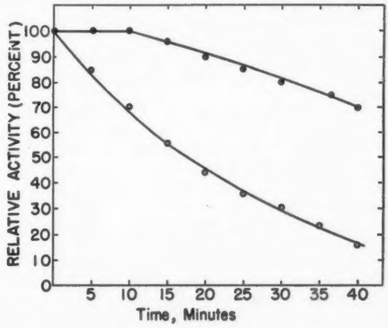


FIG. 5. Thermal stability of asparagine deamidase of *B. coagulans* (○) and *B. stearothermophilus* (●) in absence of substrate at 55° C. Conditions as described in the text. The initial protein concentration of both preparations was adjusted to the same level (0.27 mg. protein per ml.).

83% of its activity after 40 minutes at 55° C. and the enzyme from *B. stearothermophilus* lost 29% of its activity under identical test conditions. The relative instability of these preparations at 55° C. is similar to the findings of Allen (1), who reported that certain enzymes from thermophilic bacteria are not stable at 55° C. in the absence of substrate. In such cases substrate must protect the enzyme in some way from thermal inactivation.

With respect to thermal stability it appears that the enzymes of thermophilic bacteria can be placed into the following general groups: (*a*) the intracellular and extracellular enzymes that are stable at the temperature of production (usually 55° to 65° C.) but are inactivated at slightly higher temperatures (13, 14, 15, 20, 22, 23, 24); (*b*) the intracellular enzymes that are inactivated at the temperature of production in the absence of substrate as reported here and by Allen (1) and Militzer and Burns (21); and (*c*) the highly heat resistant extracellular enzymes such as α-amylase (3, 4, 8).

Considerable research is needed on the physical and chemical properties of these various enzymes before the reasons for these differences in relative thermal stability can be made clear. In this connection the recent work of Koffler *et al.* (10) on the relative thermostability of cytoplasmic and flagellar proteins of certain mesophilic and thermophilic bacteria is of interest.

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STUDY OF A PLAQUE VARIATION OF BACILLUS MEGATERIUM PHAGE[1]

H. L. EHRLICH AND C. J. PFAU[2]

Abstract

A variation in plaque type of *Bacillus megaterium* phage ψ16Bcl is described. The host culture for this study was an asporogenic strain of *B. megaterium* No. 1. The investigation revealed that the cause of the variation is lysogenization, which gives rise to two variants called *M*-1 and *M*-2. Some of their characteristics are described. The results suggest that the parent-type phage perpetuates itself during lytic cycles of reproduction, and gives rise to variant phage when it lysogenizes the host.

Introduction

During the last year, several workers have reported the occurrence of variations among temperate megaterium phages. These variants may show new plaque types, changed host ranges, or occasionally, loss of temperateness. Ionesco (2) observed that lysogenic *Bacillus megaterium* 91 (1+)° mutated after several years of stability, giving rise to two new phage types. One of these phage strains produced large, hazy plaques and the other large, clear plaques. These new phages were the result of a mutation of the lysogenic culture, which as a consequence no longer produced the 1+ type phage but instead two new types, which upon further study were found to give rise to two additional plaque-type mutants.

Northrop and Murphy (3) noted that new phage types were produced by *B. megaterium 899a* and derived substrains as a result of a change from peptone medium to synthetic medium.

Stolp (4), after isolating a phage-sensitive, sporogenous strain from lysogenic *B. megaterium 899* (H+)°, found that the new strain gave rise to plaque-type variations when the free phage from the lysogenic culture was grown on it. That the host was implicated in the production of these new types is suggested by Stolp's observation that the phage-sensitive "mutilat" strain from the *899* culture did not give rise to plaque-type variations by the H+ phage.

The present paper reports a new type of plaque variation in megaterium phage, which is not the result of spontaneous mutation of the phage.

Materials and Methods

In the majority of the experiments to be reported, an asporogenic strain of *B. megaterium* No. 1 was used as host.

The following phage strains, classified by plaque type, were studied (Figs. 1, 2). The parent strain ψ16Bcl, originally isolated in this laboratory,

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forms a large, clear plaque (about 6 mm. in diameter), which may develop a slight halo at an advanced age. Variant strain *M*-1 forms an unstable plâque that initially has a clear center surrounded by a halo, but later very often develops one or more clear, lunar sectors at the outer rim of the halo. Finally, strain *M*-2 forms stable plaques (about 3 mm. in diameter), consisting of a clear center surrounded by a halo. These plaque types are observed only on the asporogenic host. When these phage strains are grown on sporogenous parent of *B. megaterium* No. 1, the variant plaques and those of ψ16Bcl phage are very similar in appearance. The genotype of the phage strains is preserved, however.

Stock preparations of all phage strains were prepared by picking a well isolated plaque to 2 ml. sterile broth and leaching the phage content from it at about 4° C. for several hours. Agar debris, etc., were removed by transferring the supernatant to about 10 ml. of sterile broth after centrifugation. Each preparation was titered and simultaneously tested for purity with respect to plaque type. This same procedure was used for analysis of experimental plaques.

Phage titrations and plaque analyses were made by the method of Hershey *et al.* (1). The basal agar for these assays consisted of 5 g. Bacto tryptone, 3 g. Bacto beef extract, and 10 g. Bacto agar in 1 liter of distilled water. The capping agar consisted of basal agar diluted to 0.7 of the original concentration. Phage dilutions were made in broth containing 5 g. Bacto tryptone and 3 g. Bacto beef extract per liter of distilled water. This broth medium was also used for liquid culture experiments.

Results and Discussion

When morphologically pure ψ16Bcl plaques were picked individually to 2 ml. of broth suspensions of asporogenic *B. megaterium* No. 1 and incubated at 30° C. for about 24 hours, the resultant free phage, when plated out, usually gave rise to ψ16Bcl plaques and variant plaques. In a typical experiment, in which 20 plaques were picked in this manner, only one culture did not produce detectable variant phage, and four cultures produced variant phage in excess of the parent type. Pure ψ16Bcl phage was recovered when morphologically pure ψ16Bcl plaques were leached into broth in the absence of any host cells. It seemed that something happened during multiplication of the ψ16Bcl phage in broth cultures of host strain that led to the formation of the variant phages.

The possibility was considered that the variants arose as a result of lytic phage growth-cycles during which spontaneous mutation and selection of phage took place. Thus a test for spontaneous mutation was devised, based on the observation of a high frequency of occurrence of variant phage after multiplication of ψ16Bcl phage, which suggested a high mutation rate and a high selectivity for the variant. Two milliliters of a bacterial broth suspension, containing in the order of 10^5 chains of cells per ml., were introduced into each of 10 sterile vials (10×75 mm.). Next 0.1 ml. of a ψ16Bcl phage

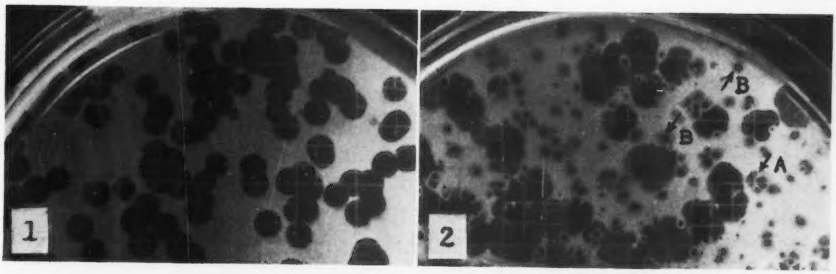

FIG. 1. Plaques of megaterium phage ψ16Bcl.
FIG. 2. Plaques of megaterium phage variants and the ψ16Bcl parent. A, variant *M*-1; B, variant *M*-2. The large plaques represent phage ψ16Bcl, here showing the slight halo which develops upon aging.

suspension, diluted to contain between 1 and 10 phages, was added to each of the 10 vials. This phage preparation was previously shown to contain less than one possible variant-phage particle per 100 ψ16Bcl particles. Thus chances of simultaneous inoculation of ψ16Bcl and variant particles into any one vial were less than 1 in 10. The content of each tube was now mixed, and all tubes were incubated in a water bath at 30° C. for about 12 hours. At the end of this time the content of each tube was plated for plaques. The results never showed any variant plaques that could be attributed to spontaneous mutation. The few variant plaques that did arise on a few plates did not follow any dilution sequence and thus must have originated on the plate and not in the vial. A 10^4- to 10^6-fold phage increase was noted in 6 to 8 out of 10 vials per experiment. Spontaneous mutation of the ψ16Bcl phage was thus ruled out as a cause of the observed variation.

As an alternative explanation of variant formation, lysogenization was considered as a possibility. To test this, plaque plates were prepared with low dilutions of ψ16Bcl phage (final titer 10^5 per ml.) mixed with host cells so that upon incubation at 30° C. for about 14 hours confluent lysis would be observed on the plates. Upon further incubation for another day or two, well isolated bacterial colonies would arise, which could be shown to consist of lysogenized host strain. In one such experiment, 15 colonies were picked randomly from the total population on a confluent lysis plate and subcultured individually. Of these 15 cultures, five were serially transferred twice more. Then the free phage associated with each of the five cultures was analyzed. It was found to consist of variant phage in association with some ψ16Bcl phage. Thus it becomes clear that lysogenization is, indeed, responsible for the variants. The formation of the variants by lysogenization is dependent upon phage–cell input ratio. A ratio of about one or greater appears to be needed (Table I, Experiment A). A requirement for a minimal host cell

TABLE I

EFFECT OF PHAGE–CELL INPUT RATIO ON VARIANT-PHAGE FORMATION

Experiment	Phage input $\times 10^6$	Cell input $\times 10^5$	Input ratio	Phage yield $\times 10^6$	
				Bcl	*M*-1
A	17.5	14.5	12.1	335	145
	17.5	145	1.21	59	2
	0.175	145	0.0121	59	0
	0.00175	145	0.000121	49	0
B	3.7	1.9	19.4	129	8
	1.8	1.9	9.7	172	7
	1.2	1.9	6.3	128	7
	0.9	1.9	4.6	155	4
	0.4	1.9	1.9	172	7
	3.7	9.5	3.8	103	32
	3.7	19.0	1.9	320	82

NOTE: Input numbers of phages and cells each contained in 1 ml. of broth. They were added to 8 ml. of sterile broth, incubated at 30° C. for approximately 24 hours, and then assayed for free phage.

concentration is also suggested by the data in Experiment B of Table I. The frequency of lysogenization by ψ16Bcl as measured in terms of host survival of phage infection is optimally of the order of 1 in 10^4 chains of cells. The variant phage produced is also capable of lysogenization and is about twice as effective as the parent strain. This property of the variant phage undoubtedly accounts for successful competition with the parent strain when the two are infecting the host in a mixture, despite its relative adsorption rate being 0.76–0.95 times that of the parent phage.

A closer study of the variant phage revealed that it consists of two types. One type, *M*-1, gives plaques of unstable morphology and the other type, *M*-2, gives plaques of stable morphology (Fig. 2). The phage content of *M*-1 plaques as a rule consisted of *M*-1 and ψ16Bcl phages; that of *M*-2 consisted of *M*-2 and *M*-1 phages. The reason for classing the two types as distinct from each other is that when they occur in a mixture, the proportion in which their plaques occur remains constant upon serial dilution.

Unfortunately, the susceptible host strain is a chain former. This makes it difficult to determine what happens in individual cells upon infection with any of the phage types, including the parent strain. The possibility exists that the asporogenic host strain carries a prophage naturally and that this prophage undergoes recombination with the ψ16Bcl phage during lysogenization. However, no evidence of a prophage in the host strain has been obtainable either by induction with ultraviolet irradiation or by demonstrating the liberation of free phage by use of a different indicator strain of *B. megaterium.*

The results of the experiments described suggest that ψ16Bcl phage gives rise to progeny like itself when multiplying by lytic growth-cycles, and to variant phage during lysogeny.

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SOME METABOLIC EFFECTS OF ADENOVIRUS INFECTION IN HELA CELLS[1]

K. R. ROZEE, L. J. OTTEY, AND C. E. VAN ROOYEN

Abstract

HeLa cells infected with type 7 adenovirus produced amounts of lactic acid in excess of those produced by uninfected HeLa cells, as has been found with other adenovirus types. It was found that the increased rate of glucose utilization and higher lactic dehydrogenase activity in adenovirus-infected HeLa cells could partially explain this phenomenon. It is suggested that the terminal degradative mechanisms are more active in adenovirus-infected HeLa cells and that an operative tricarboxylic acid cycle is necessary for optimum replication of type 7 adenovirus.

Introduction

It has been known for some time that infecting a cell with certain viruses initiates an increase in lactic acid production (6). Levy *et al.* (8) have shown that type 3 poliovirus causes an increase in lactic acid production within an hour after infecting monkey kidney cells. A similar phenomenon occurs with type 2 adenovirus grown in HeLa cells although the lactic acid increase does not appear as rapidly as with poliovirus infection and persists for a longer time. Fisher and Ginsberg (4) have reported that type 4 adenovirus infection of HeLa cells causes an increase in glucose utilization and concurrently an increase in lactic acid and α-keto acids.

In this laboratory type 7 adenovirus, like other members of this family, was found to increase lactic acid production after infecting HeLa cells. The work reported here is an attempt to ascertain the reasons for the increased lactic acid production in this system and to study the effect of type 7 adenovirus infection upon some metabolic processes in HeLa cells.

Methods and Results

General

HeLa cells used in the following experiments were obtained originally from Microbiological Associates Inc., Bethesda, Maryland. This cell line has been kept by us for over a year in Roux bottles on a medium used for routine cultures, consisting of 20% human serum in Scherer's maintenance medium (11).

Seven- to ten-day-old cultures of HeLa cells in Roux bottles were dispersed with 0.25% trypsin, washed twice, and diluted with routine medium (above) so as to contain approximately 6×10^4 cells per ml. This HeLa cell suspension, in 2.5 ml. aliquots, was placed in T-flasks and incubated at 37° C. until the flasks were used, usually from 6 to 10 days from the time of inoculation. The fluid over the T-flask cultures was renewed every 2 to 3 days as required.

[1] Manuscript received July 12, 1957.
Contribution from the Department of Bacteriology, Dalhousie University, Halifax, Nova Scotia.

The type 7 adenovirus was a strain originally isolated from service personnel with respiratory illness (2) and was kindly supplied by Dr. M. R. Hilleman of Walter Reed Army Institute of Research, Washington, D.C.

Glucose Utilization Experiments

The following is a typical experiment. Four cultures of HeLa cells in T-flasks were infected on the 6th day with 50 times the tissue culture infective dose giving 50% infection ($TCID_{50}$) type 7 adenovirus per ml. The medium contained 15% rabbit serum in M 202 (a complete medium similar to 199) with the glucose concentration increased to 3 mg./ml. Four identical but uninfected cultures in the same medium were used as controls.

Samples of medium were removed from all eight flasks for glucose determination at intervals. The original volume was kept constant by replacing the medium removed with medium from one treated or one control flask in the same set. Glucose was determined by the Nelson–Somogyi method (12) and the results of a typical experiment are given in Fig. 1. The variation of individual determinations was within 8% of the mean and each point given is the mean of at least three determinations.

From Fig. 1 it is apparent that the infected cells take up glucose initially at a rate almost a third greater than do the uninfected cells. The infected cell rate is maintained for the first 24 hours and thereafter decreases until at the end of 60 hours the rate is about one-half that of the uninfected cells.

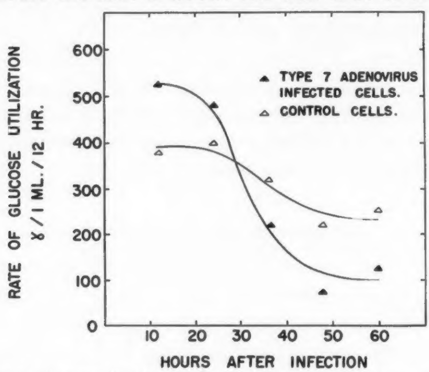

FIG. 1. The effect of type 7 adenovirus infection on glucose utilization by HeLa cell cultures.

Fluoroacetate Experiments

Sodium fluoroacetate has been shown to be a fairly specific inhibitor of the tricarboxylic acid cycle acting at the aconitase level (10). Experiments were designed to show the effect of fluoroacetate on the ability of HeLa cells to support adenovirus reproduction.

To be assured that a block was occurring after the addition of fluoroacetate, citric acid levels were measured by the method of Natelson *et al.* (9) in fluoroacetate-treated and control cells. Citric acid levels were also determined on cells which had been infected with 50 $TCID_{50}$ type 7 adenovirus per ml. with

or without fluoroacetate present. All experiments were carried out with cells maintained on 15% rabbit serum in Scherer's medium. The citric acid levels were determined 5 days after the addition of fluoroacteate or fluoroacetate plus type 7 adenovirus. The HeLa cells were disrupted by freeze-thawing immediately before they were assayed.

The citrate accumulation in fluoroacetate-treated cells indicates that blocking of the tricarboxylic acid has occurred.

The low amount of citrate found in adenovirus-infected HeLa cells and the greater accumulation of citrate in these cells after fluoroacetate poisoning suggests that the tricarboxylic acid cycle operated at an increased rate after adenovirus infection. To ascertain the importance of this cycle to virus reproduction the following experiment was carried out.

Two cultures of HeLa cells after 7 days in Roux bottles were washed several times in Scherer's medium. One bottle was infected with 50 $TCID_{50}$ type 7 adenovirus per ml. and the second was infected with 50 $TCID_{50}$ type 7 adenovirus per ml. and also inhibited with 5×10^{-3} *M* sodium fluoroacetate. At the end of 6 days both cultures were frozen and thawed three times, and the viral activity of these preparations was assayed by the method of Reed and Meunch. The results of a typical experiment are recorded in Table II. It can be seen that the control HeLa cell cultures produced a virus titer 300 times greater than did the fluoroacetate-inhibited HeLa cell culture.

Pyruvate Levels in Adenovirus-Infected Cells

Five T-flask cultures of HeLa cells, 6 days old, were washed three times with Scherer's medium, modified so as to contain 3 mg./ml. glucose and with pyruvate omitted. The cultures were then infected with 50 $TCID_{50}$ type 7 adenovirus per ml. in 15% rabbit serum and modified Scherer's medium. Five identical cultures, treated the same but uninfected, served as controls. Cultures were removed at intervals, frozen, and were later assayed for pyruvate by the method of Friedemann and Haugen (5). The results are presented in Fig. 2.

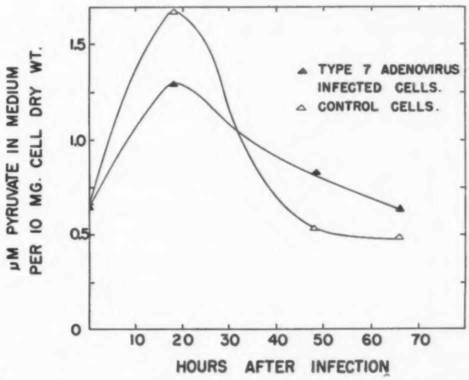

FIG. 2. The effect of type 7 adenovirus infection on pyruvate metabolism in HeLa cell cultures.

Lactic Dehydrogenase Levels

Since the adenovirus–HeLa cell system initially produced more lactic acid than was produced by uninfected HeLa cells it was of interest to compare their levels of lactic dehydrogenase activity.

Eight Roux bottles containing HeLa cultures, 7 days old, were infected with 500 $TCID_{50}$ type 7 adenovirus per ml. in 15% rabbit serum and Scherer's medium. Eight uninfected Roux-bottle cultures served as a control. After 48 hours the cell sheets were dispersed by trypsin (0.25%), washed twice in phosphate-buffered saline pH 7.6 (3), and resuspended in 20 ml. of the buffered saline. This constituted approximately a 4.0% cell suspension.

The adenovirus-infected cells and the uninfected controls were then disintegrated with a Raytheon 9 kc. sonic oscillator for 20 minutes. The disrupted cells were then frozen and later assayed for lactic dehydrogenase by the colorimetric method of Kun and Abood (7). The results are shown in Table III and indicate that lactic dehydrogenase levels in adenovirus-infected cells are more than twice those found in normal HeLa control cells.

Discussion

The increased rate of glucose uptake after type 7, type 2 (8), and type 4 (4) adenovirus infection suggests that glycolytic processes are more active in all adenovirus-infected cells.

In studies with type 4 adenovirus and HeLa cells, Fisher and Ginsberg (4) found that more glucose was utilized than could be accounted for in the amount of lactic acid that accumulated in infected cells. It seems, then, that metabolites of the tricarboxylic acid cycle beyond pyruvate are further degraded in these cells.

Since it has been shown that HeLa cells contain all the enzymatic systems of the tricarboxylic acid cycle (1) it is probable that this cycle is responsible for the degradation of some of the metabolites of glucose utilization which are not accounted for in the lactic acid accumulation in adenovirus-infected HeLa cells.

The results in Tables I and II suggest that for optimum yield of virus the tricarboxylic acid cycle is required. This is apparent in the reduced titer of virus from fluoroacetate-inhibited cells (Table II) and in the increased activity

TABLE I

CITRATE ACCUMULATION IN ADENOVIRUS-INFECTED AND UNINFECTED HELA CELLS AFTER TREATMENT WITH 5×10^{-3} *M* FLUOROACETATE

Figures indicate γ citrate found per 10 mg. total organic solid and each is an average of two separate experiments

	Additions to HeLa cell culture medium			
	No additions	50 $TCID_{50}$ adeno-7 per ml.	5×10^{-3} *M* FAc	50 $TCID_{50}$ adeno-7 per ml. + 5×10^{-3} *M* FAc
γ Citric acid	6.6	2.1	14.6	18.5

of the tricarboxylic acid inferred from Table I. This increased activity is, of course, presumptive but explains the very low citrate levels in infected cells and the high levels found in infected cells inhibited by fluoroacetate. The presumption seems plausible in view of the increased rate of glucose uptake in infected cells as shown in Fig. 1.

TABLE II

THE EFFECT OF 5×10^{-3} *M* FAc ON ADENOVIRUS TYPE 7 REPRODUCTION IN HeLa CELLS

The titration method has an accuracy in this laboratory of ± 0.6 log and each determination is an average of two separate experiments

	Additions to HeLa cell culture medium	
	Adeno-7 50 $TCID_{50}$/ml.	Adeno-7 50 $TCID_{50}$/ml. $+ 5 \times 10^{-3}$ *M* FAc
$TCID_{50}$ per ml. 6 days at 37° C. after additions	$10^{7.2}$	$10^{5.0}$

As can be seen from Table III, the lactic dehydrogenase activity of adenovirus-infected cells is greater than the normal uninfected control HeLa cells. Considering this, it would be expected that the pyruvate levels in infected cells should be lower than in uninfected cells.

TABLE III

THE EFFECT OF TYPE 7 ADENOVIRUS INFECTION ON THE ACTIVITY OF LACTIC DEHYDROGENASE IN HeLa CELLS

The system consisted of 2.0 ml. sonicate, 0.1 mg. DPN, 10 mg. KCN, 0.2 *M* lactic acid, and 4 mg. 2,3,5-triphenyl-(2H)-tetrazolium chloride made up to 4.5 ml. in 0.05 *M* phosphate buffer pH 8.0; two experiments are reported, one incubated at 35° C. for 24 hours and the other for 16 hours before determination of the Δ O.D.

Disrupted cell preparation	Normal HeLa cells	Adenovirus-infected HeLa cells, 48 hr. postinfection	Ratio $\frac{\text{infected}}{\text{normal}}$
Δ O.D. per 5 mg., 16 hr.	0.055	0.1080	1.92
Cell dry wt. at 445 mμ, 24 hr.	0.1620	0.420	2.59

As indicated in Fig. 2, the pyruvate level maintained by infected HeLa cells is considerably lower than that maintained by uninfected cells until 24 to 30 hours after infection. Thereafter the infected cells tend to accumulate more pyruvate than the controls. The increase in rate from the start of the experiment, when the cells were transferred from human serum to rabbit serum, until roughly 20 hours later may have been caused by the change of medium, bearing in mind that these cells were routinely grown on human serum.

These observations, especially those concerning the tricarboxylic acid cycle and lactic dehydrogenase activity, may indicate that changes in glucose metabolism attendant upon adenovirus infection in HeLa cells are due to

direct virus intervention rather than to nonspecific degenerative changes accompanying infection. Since infection with other viruses seems to initiate changes which are dissimilar to those seen with adenoviruses (4), it would be of some interest to determine if reproduction of viruses belonging to other unrelated groups is similarly affected by inhibitors of the tricarboxylic acid cycle in host cells.

Acknowledgments

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ANTIGENIC ANALYSIS OF DISSOCIANTS AND SEROLOGICAL TYPES OF PASTEURELLA MULTOCIDA[1]

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Abstract

Pasteurella multocida has been separated into serological types on the basis of a variety of immunological methods by earlier workers. A definite dissociation pattern has also been described. This study presents evidence that two colonial variants of a type 1 strain are qualitatively identical in their antigenic structure but differ quantitatively with regard to one antigen. The data also indicate the similarity of somatic antigens of three type strains though antigenic differences can be demonstrated in the Jennings triangle plate which possibly represent differences in the substances composing the capsule.

Introduction

Early workers did not recognize the unitarian nature of the etiological agent of hemorrhagic septicemia, *Pasteurella multocida*, but rather believed in the existence of distinct species within the group, based on the zoologic source of isolation; however, during this early period observations of dissociation patterns and serological distinctions were recorded. Rosenbusch and Merchant (13), attempting to correlate the literature, (1) presented data which indicated the fallacy in the zoologic classification and suggested *P. multocida* to include those organisms comprising this classification, (2) studied cultural variation in conjunction with virulence, and (3) proposed a classification of this organism into subgroups on the basis of agglutination reactions and fermentation of xylose, arabinose, and dulcitol. Little and Lyon (9), employing passive mouse immunization and rapid slide agglutination, established a classification consisting of three serological types. Roberts (12) used the criterion of cross-protection in mice as a basis for a categorization of this bacterium into at least four distinct types. Carter (3) showed the presence of type specific capsular material and established a serological grouping using precipitin tests and capsular swelling techniques. Carter and Byrne (4) related Carter's system to those proposed by the previous investigators. By means of a hemagglutination method, which he states is more sensitive, Carter (2) re-evaluated the correspondence of the four classifications. Priestley (11) studied the nature of the capsule, indicating its relationship to virulence, and demonstrated the presence of a somatic antigen common to both virulent and avirulent, i.e., encapsulated and unencapsulated strains. He also gave evidence of differences in other somatic antigens. Carter (3), however, stated that the somatic antigenic structure consists of a single component.

Using a great many strains isolated from cases of fowl cholera, Hughes (7) described three types of colony formation on hemoglobin agar when viewed with oblique light: a large, "fluorescent" type (*F*) composed of highly virulent

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Contribution from the Department of Biological Sciences, University of Delaware, Newark, Delaware, U.S.A. This investigation was supported by the University of Delaware Research Foundation.

organisms which did not agglutinate in homologous antiserum; a smaller, nonfluorescent "blue" type (*B*) formed by highly agglutinable organisms of low virulence; and an "intermediate" type (*I*) whose properties varied between those of the other types. Hughes studied other properties of these colonial variants and discussed their dissociation pattern.

Elberg and Chen-Lee Ho (6) studied the dissociation pattern in detail, relating it to virulence, and demonstrated antigenic strain differences. Yaw *et al.* (14) showed the virulence for two hosts of the dissociants of three serological types.

In a recent paper Carter (1) studied the relationship between colony type and antigenic characteristics as determined with acriflavine and proposed the terms mucoid, smooth, and rough be applied to the colonial variants.

Yaw and Kakavas (15) demonstrated that immunization with the polysaccharide capsule obtained from the *F* variant of type 1 strain protected both mice and chickens from homologous challenge while a vaccine consisting of cells of the *B* variant of type 1 protected chickens but did not protect mice. This suggested that the presumably unencapsulated variant and the capsular material might possess similar protective antigens, differing primarily in quantity as suggested by the difference in protection of mice and chickens, the chicken being able to engender protective antibodies by a stimulus from the small quantity of antigen present in the unencapsulated variant.

The literature thus indicates that the pathogenicity of *P. multocida* for particular hosts might be dependent upon the presence and concentration of the type specific polysaccharide capsule (11). The purpose of this research was to compare the antigenic structure of the *F* and *B* variants of a type 1 strain in an attempt to correlate virulence with the presence and location of particular antigenic components and to demonstrate the antigenic differences in the various serological types.

Materials and Methods

A representative culture of each of the serological types of Little and Lyon (9) was used in this study. The strains used were: type 1, 9137; type 2, 2872; and type 3, 6355 (14). A description of the *F* and *B* variants of the type 1 strain used is given in Table I. Trypticase Soy (TS) agar or broth (Baltimore Biological Laboratory) was the medium employed throughout this study.

The detection of individual somatic and capsular antigens required a method enabling the visual separation of these components. For this purpose the serum–agar technique of Jennings (8) was chosen. The protocol of the method as modified by London and Kakavas (10) was followed with two exceptions: the bacterial cells were grown on 30 agar plates, the growth removed with a large rubber spatula, and grinding accomplished in a short time at room temperature by substituting levigated alumina (2.5$\times$wet weight of cells) for powdered carborundum. The ground cells were suspended

in 9 ml. physiological saline containing Merthiolate in a concentration of 1:10,000. Particulate matter, i.e., cellular debris and the levigated alumina, was removed by centrifugation. Several lots of precipitinogens were prepared as described and found to give comparable zone patterns in the triangle plate.

TABLE I

CHARACTERISTICS OF THE *F* AND *B* COLONIAL VARIANTS OF *Pasteurella multocida* (Type 1 strain)

Characteristic	*F* variant	*B* variant
Mouse virulence	Less than 10 organism kill	Relatively avirulent—approximately 10^6 cells required to kill
Stability of dissociant	Unstable—frequent reisolation necessary	Stable for 3 years
Colony appearance on TS agar, viewed with oblique light	Reddish "fluorescence"	Uniformly blue
Colony size	Large	Smaller
Stability in physiological saline	Stable	Autoagglutinates
Capsule	Encapsulated	Capsule not detectable
Agglutinability	Does not agglutinate in homologous antiserum	Agglutinates in antiserum

NOTE: Virulent *F* variant passed through mice prior to preparation of antigens.

The polysaccharide capsular precipitinogens of 9137 were prepared by suspending the growth from 30 Petri plates in 9 ml. physiological saline, filtering through cotton to remove agar particles, heating for 30 minutes at 56° C., and removing particulate matter by prolonged centrifugation.

Antisera were produced in chickens, mice, and rabbits using bacterial cells suspended in formalized (0.2%) physiological saline, turbidity corresponding to a McFarland nephelometer No. 3. Mice were given 9 or 12 intraperitoneal injections of 0.2 ml. over a period of 3 or 4 weeks, antisera from 10 individuals being pooled. Chickens were immunized by three or four intramuscular injections of 0.2 ml. of antigen. The schedule of each rabbit immunized was varied depending upon the reactivity of the antisera from trial bleedings. Whenever possible, blood was taken (by cardiac puncture) several times from each rabbit in order to obtain antisera of different sensitivities. The antisera were preserved with Merthiolate (1:10,000) and stored in the refrigerator in sealed tubes.

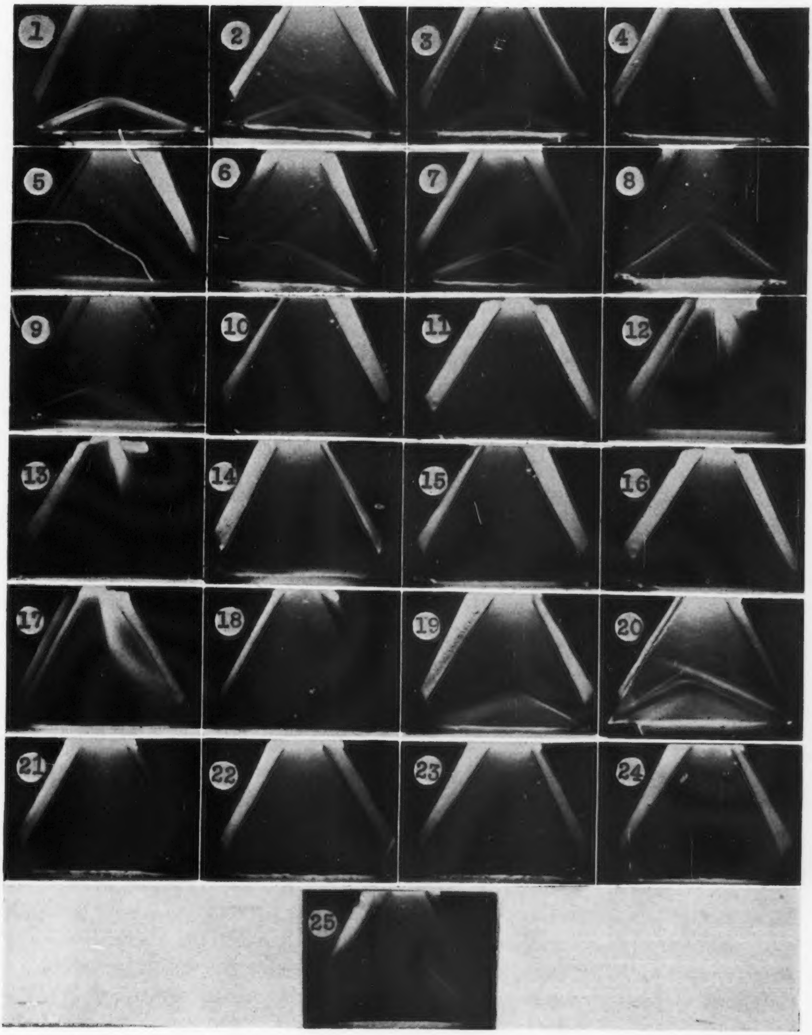
Results

The zone patterns observed in the Jennings triangle plate with various combinations of precipitinogens and precipitins are presented in Figs. 1–25. The reactants contained in each reservoir are given in the accompanying key. Only the central reaction arena is shown in the photographs; the rest of the triangle plate was masked during the photographing. The possibility of antibodies directed against agar, which might have been present in the antigen preparation used for immunization, was eliminated by absorption experiments.

The first phase of this work was concerned with the direct comparison of the *F* and *B* variants of strain 9137 in order to determine the minimal number of antigenic substances composing these organisms. The zone patterns shown in Figs. 1–3 indicate that at least six antigenic substances are found in both variants and that various 9137*F* antisera and 9137*B* rabbit antisera are not able to distinguish any antigenic differences between the *F* and *B*

FIGS. 1–25. Zone patterns in the Jennings plate with various *Pasteurella multocida* precipitins and precipitinogens. Location of the source of the diffusing reactants is indicated separately for each figure in the following order: upper left reservoir; upper right reservoir; lower reservoir. A portion of the reservoirs not covered by the mask can be seen bordering the reaction arena.

FIG. 1. 9137*F* precipitinogens; 9137*B* precipitinogens; rabbit 16*A* antiserum vs. 9137*B*. FIG. 2. 9137*F* precipitinogens; 9137*B* precipitinogens; rabbit *M* antiserum (9–21 bleeding) vs. 9137*F*. FIG. 3. 9137*F* precipitinogens; 9137*B* precipitinogens; rabbit *J* antiserum (8–22 bleeding) vs. 9137*F*. FIG. 4. 9137*F* precipitinogens; 9137*B* precipitinogens; chicken antiserum immunized with capsular polysaccharide and challenged with 9137*F*. FIG. 5. 9137*F* precipitinogens; 9137*B* precipitinogens; mouse antiserum vs. 9137*B*. FIG. 6. Capsular polysaccharide; 9137*B* precipitinogens; rabbit *M* antiserum (9–4 bleeding) vs. 9137*F*. FIG. 7. 9137*B* precipitinogens; capsular polysaccharide; rabbit 11 antiserum (11–5 bleeding) vs. 9137*F*. FIG. 8. Rabbit 12 antiserum (11–12 bleeding) vs. 9137*F*; rabbit 16*A* antiserum (11–14 bleeding) vs. 9137*B*; capsular polysaccharide. FIG. 9. Capsular polysaccharide; 9137*B* precipitinogens; rabbit 11 antiserum (11–12 bleeding) vs. 9137*F*. FIG. 10. 9137*F* precipitinogens; 9137*B* precipitinogens; rabbit *J* antiserum (8–22 bleeding) vs. 9137*F* absorbed with 9137*B* precipitinogens. FIG. 11. 9137*F* precipitinogens; 9137*B* precipitinogens; rabbit *M* antiserum (8–22 bleeding) vs. 9137*F* absorbed with 9137*B* precipitinogens. FIG. 12. Rabbit *M* antiserum (8–22 bleeding) vs. 9137*F* absorbed with 9137*B* precipitinogens; rabbit *M* antiserum (8–22 bleeding) vs. 9137*F*; capsular polysaccharide. FIG. 13. Rabbit *J* antiserum (8–22 bleeding) vs. 9137*F* absorbed with 9137*B* precipitinogens; rabbit *J* antiserum (8–22 bleeding) vs. 9137*F*; capsular polysaccharide. FIG. 14. 9137*F* precipitinogens; capsular polysaccharide; pooled antisera from rabbits 11 and 12 (11–12 bleeding) vs. 9137*F* absorbed with 9137*B* precipitinogens. FIG. 15. 9137*F* precipitinogens; 9137*B* precipitinogens; rabbit *J* antiserum (8–22 bleeding) vs. 9137*F* absorbed with 2872*I* precipitinogens. FIG. 16. 9137*F* precipitinogens; 9137*B* precipitinogens; rabbit *M* antiserum (8–22 bleeding) vs. 9137*F* absorbed with 2872*I* precipitinogens. FIG. 17. Rabbit 12 antiserum (11–12 bleeding) vs. 9137*F* absorbed with 2872*I* precipitinogens; rabbit 12 antiserum (11–12 bleeding) vs. 9137*F*; 9137*F* precipitinogens. FIG. 18. 2872*I* precipitinogens; 9137*B* precipitinogens; rabbit 10 antiserum vs. 2872*I* absorbed with 9137*F* precipitinogens. FIG. 19. 9137*F* precipitinogens; 6535*F* precipitinogens; rabbit *M* antiserum (8–22 bleeding) vs. 9137*F*. FIG. 20. 6535*F* precipitinogens; 9137*F* precipitinogens; rabbit 14 antiserum vs. 6535*F*. FIG. 21. 6535*F* precipitinogens; 9137*B* precipitinogens; rabbit 14 antiserum vs. 6535*F* absorbed with 9137*F* precipitinogens. FIG. 22. 2872*I* precipitinogens; capsular polysaccharide; rabbit 14 antiserum vs. 6535*F* absorbed with 9137*F* precipitinogens. FIG. 23. 6535*F* precipitinogens; capsular polysaccharide; rabbit 4 antiserum vs. 2872*I* absorbed with 9137*F* precipitinogens. FIG. 24. 2872*I* precipitinogens; capsular polysaccharide; rabbit 7 antiserum vs. 6535*F* absorbed with 9137*F* precipitinogens. FIG. 25. Rabbit 12 antiserum (11–6 bleeding) vs. 9137*F*; rabbit 12 antiserum (11–6 bleeding) vs. 9137*F*; purified capsular polysaccharide.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dissociants of this strain. The variation in zone pattern is a characteristic of the particular serum employed. Chicken antiserum shows four similar zones from *F* and *B* of strain 9137 (Fig. 4). Mouse antiserum is not able to distinguish between these variants as is shown in Fig. 5, at least two zones from both antigen sources being present.

Since several closely situated zones were observed, the possibility of superimposition of zones was considered. It was thought that by comparing 9137*B* with the capsular polysaccharide of 9137*F*, the complexity of the zone pattern would be lessened, since the capsular polysaccharide contains only a portion of the cell's antigenic substances; also, any similarities between the non-capsulated variant and the specific capsular polysaccharide of this type 1 strain would be seen. Figs. 6–9 indicate that zones produced by the capsular polysaccharide in all cases coalesce with certain of the zones elicited by the 9137*B* antigens. To eliminate differences in the ability of individual rabbits to make antigenic distinctions, antisera from several rabbits and different bleedings of the same rabbit were used. Throughout this study the capsular polysaccharide never produced any zones which did not show identity with certain 9137*B* zones. The results seemed to indicate that although a capsule surrounding 9137*B* cells could not be observed with a compound microscope, this variant does possess antigens similar or identical with those found in the capsule of 9137*F*. Chicken and mouse antisera gave comparable results.

To establish more conclusively the qualitative similarity of the two variants, precipitin absorption was used. A quantity of 9137*B* cells was ground with levigated alumina and then suspended in a few milliliters of 9137*F* antiserum. The suspension was mixed on a shaking device for several hours. The antiserum was separated from the absorbing material by centrifugation and stored in the refrigerator in sealed tubes. Completeness of absorption was determined by reacting the absorbed antiserum with 9137*B* precipitinogens in the Jennings plate. Several lots of antisera were absorbed in this manner and then reacted with combinations of 9137*B*, 9137*F*, and the capsular polysaccharide. The resulting zone patterns are presented in Figs. 10–14. The zones originating from the apex of the triangle in Figs. 12 and 13 resulted from an excess of absorbing precipitinogens in the absorbed antiserum reacting with the unabsorbed antiserum. The complete absence of zones with the absorbed antisera provides additional evidence for the identity of 9137*F* and 9137*B* antigens, since 9137*B* can remove all antibodies for 9137*F* antigens including those associated with the capsule. Reciprocal absorption with 9137*B* antiserum and 9137*F* precipitinogens gives comparable results.

The lack of a demonstrable capsule in 9137*B* plus the fact that two variants of this type 1 strain possess the same antigens suggest a quantitative difference in the antigenic structure. This proposed quantitative difference was tested for in the following manner. Antiserum prepared against 9137*F* was absorbed completely with an excess of ground 9137*B* cells as described previously except that the absorbing material was packed by centrifugation but not removed from the antiserum. To this absorbed antiserum plus absorbing material was added a small quantity of unabsorbed 9137*F* antiserum.

After a few hours on the shaker, the material in the tube was centrifuged to pack the cellular material and 0.5 ml. of the antisera removed and tested in the Jennings plate against 9137*F* and 9137*B*. This procedure was repeated four times before any zones were observed. The purpose of this procedure was to prepare an antiserum which contained a small amount of the antibody formed against the antigen(s) found in greater concentration in 9137*F* cells. The result of this experiment was the appearance of a very faint zone initiated by 9137*F* antigens while no zone was formed with 9137*B* precipitinogens. This indicates, if the above reasoning is correct, that 9137*F* and 9137*B* do differ quantitatively with respect to the antigen associated with the capsular polysaccharide of 9137*F*. With the concentration of antiserum routinely used, no differences were observed in displacement or number of zones when undiluted capsular polysaccharide was compared with a dilution of 1:2. Hence, large concentration differences could remain undetected with the routinely used antiserum concentration but can be distinguished with a very small amount of antibody as outlined above. Owing to the slight intensity of this zone, satisfactory photographs were not obtainable.

The second phase of this study involved the use of different antisera absorbed with various precipitinogens to detect differences in three serological type strains of *P. multocida*. The procedure followed has been described above. The first comparison was made between 2872*I* and 9137*F*; four different 9137*F* sera were absorbed with ground cells of 2872*I* and reacted with 9137*F* and/or 9137*B*. Figs. 15–17 represent the observed zone patterns. In Fig. 17 the zones extending from the apex of the triangle resulted from excess absorbing antigen reacting with unabsorbed sera.

Fig. 15 shows one zone arising from the 9137*F* antigen source and no zone from 9137*B*, while Fig. 16 shows the same zone and an additional one arising from both 9137*F* and 9137*B*. The added zone in Fig. 16 is probably due to the ability of the particular antiserum to make a further distinction between types 1 and 2. The data thus indicate that types 1 and 2 possess several antigens in common and that type 1 contains at least two antigens not found in type 2. The presence of the small zone from the 9137*F* side and not from the 9137*B* side (which appears identical with the zone patterns described above in the experiment performed to determine quantitative relationships) provides further evidence for the quantitative difference between the variants of type 1, and might indicate that a quantitative difference exists in the concentration of an antigen shared by types 1 and 2, namely the capsular polysaccharide.

When 2872*I* antiserum absorbed with ground 9137*F* was reacted with the homologous antigen and 9137*B*, the zone pattern depicted in Fig. 18 was observed. The 9137*F* precipitinogens absorbed out all antibodies to 9137*B*, as was expected, but did not remove all the antibodies directed towards 2872*I* antigens not found in type 1. Direct comparisons of types 1 and 2 were made by reacting 2872*I* antiserum and separately 9137*F* antiserum with the precipitinogens of 2872*I* and 9137*F* in the Jennings plate. Discernible zone patterns could not be photographed owing to the complexity of the zone

patterns; however, these patterns appeared to show an antigen in each type not found in the other. The results with absorbed antisera would seem to substantiate these differences.

The next comparison was made between types 1 and 3. Both direct comparison with unabsorbed sera and comparison with absorbed sera were used. The zone patterns presented in Figs. 19 and 20 show that 9137*F* and 6535*F* each possess an antigen not in common with the other, the majority of antigens being shared. In Fig. 19 it is noted that a thin, outermost zone occurs between the antiserum reservoir and the homologous 9137*F* precipitinogen source. Since the tip of this zone shows bending rather than proceeding directly across the reaction arena, the antigen responsible for its presence is not completely exclusive for 9137*F*. This bending of the zone is believed to indicate the presence from both antigen sources of similar molecules, the lack of appearance of the comparable zone from 6535*F* being due to an insufficient concentration of the particular antigen. The other zone from 9137*F* and the additional zone from 6535*F* in Fig. 20 do not show significant bending, thus indicating a unique antigen in each case.

The two zones arising from 6535*F* and the homologous antiserum absorbed with 9137*F* shown in Fig. 21 give added visual evidence of differences between types 1 and 3. That two zones are observed in Fig. 21 and apparently only one additional zone in Fig. 20 cannot be adequately explained at this time.

In Figs. 22–24, it is seen that after absorption with 9137*F*, two samples of 6535*F* antisera retain an antibody directed towards an antigen found in 2872*I*, and an antiserum against 2872*I* retains an antibody for a 6535*F* antigen. The position and intensity of the zones would seem to indicate that the eliciting antigen not found in type 1 is shared by types 2 and 3. A direct comparison of types 2 and 3, i.e., antiserum against each type reacted with precipitinogens of both types, revealed an additional antigen in 2872*I* and in 6535*F* not shared by the other type. Distinct photographs of these comparisons were not obtainable.

It should be mentioned that the above results with absorbed antisera were not obtained when 2872*I* and 6535*F* antisera from other rabbits were employed. In these instances no zones were observed between the absorbed antisera and their homologous precipitinogens, i.e., no differences between types 1 and 2 and between types 1 and 3 were found. This might possibly indicate that, since one rabbit could make an antigenic distinction while another similarly immunized one could not, the additional antigen found in one type has some structural relationship to an antigen present in the other type.

Fig. 25 shows the zones formed by the purified capsular polysaccharide prepared according to Carter's protocol, and 9137*F* antisera.

Discussion

The zone patterns observed with 9137*F* and 9137*B* precipitinogens and the homologous antisera clearly demonstrate at least six antigenic components. The presence of two or three antigenic substances in the purified capsular polysaccharide, as seen in Fig. 25, indicates that Carter's statement (3) of a

single somatic antigen is not valid. The present data suggest at least three surface or intracellular antigens common to both type 1 variants. As described above these antigens are also found in the strains tested representing types 2 and 3.

If it is assumed that at least one of the many rabbits immunized formed the maximal number of specific antibodies, it can be stated that the lack of a demonstrable capsule in 9137*B* is not indicative of the absence of the antigen associated with the capsule of 9137*F*, but rather is the result of an insufficient concentration of this substance to be observed microscopically. The authors feel that the absorption studies are additional evidence in support of this argument. The initial suggestion from the results of Yaw and Kakavas (15) can be explained on the basis of this quantitative difference. This view is in accord with the studies of Chen and Meyer (5) dealing with *Pasteurella pestis*.

Fig. 4 shows the zones resulting from the interaction of 9137*F* chicken antiserum and both variants. It is seen that the chicken cannot distinguish between 9137*F* and 9137*B*, qualitatively or quantitatively. However, the authors found chicken antiserum to react with difficulty in the Jennings plate and found several sera from immunized chickens unable to form distinct zones or in some cases any zones at all. The reliability of chicken antiserum in serum–agar studies is therefore questionable.

The various serological classifications of *P. multocida* were based on methods which could only detect exposed or capsular-type antigens. The data presented comparing the three types of this organism show that specific antigen(s) for each type is (are) present, and since the earlier workers detected specific surface antigens, those observed in this study are undoubtedly the antigens responsible for the serological typing of this species. Further work comparing purified capsular material of the three types should confirm this view.

These results suggest that the protective ability engendered by various types and the virulence of *P. multocida* may be associated with the kind and quantity of some surface substance and that the sensitivity of the particular host to this substance must be considered. They further suggest that the somatic, i.e., surface and intracellular, antigens of the strains studied are identical and thus any antigenic differences must be associated with capsular or slime layer substances.

Mouse antisera were also studied. As with the chicken antisera, several preparations of immune mouse sera did not show any visible reaction. Hemoglobin present in reactive samples of mouse antisera obscured the zone pattern too much for successful photographs but with these preparations 9137*F* and 9137*B* appeared identical.

As was noted previously, the *F* and *B* variants differ in virulence for mice by a factor of about 10^5. The authors have suggested that a concentration difference of a protective antigen(s) might account for this. However, the existence of some metabolic product in the *F* variant which is responsible for virulence has not been excluded. Further work on this aspect is needed.

An unexplainable observation, which the authors feel is significant, is the inagglutinability of the *F* variant in homologous antiserum. Since this is contrary to what occurs with most other encapsulated organisms, it is thought that an elucidation of the factors involved in this inagglutinability might indicate fundamental differences between the *F* and *B* variants and also add pertinent data regarding antigen–antibody reactions.

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CORRECTIONS

Volume 2, page 604. In Table I, "N", the last letter in the last column, should read "H".

Volume 3, page 152. To paragraph 2 should be added: Erickson (7) stated that certain strains of *Micromonospora* could utilize α-heptachloropropane but scarcely trichloroacetic acid as carbon source.

Volume 3, page 158. In Table II, "vitamin B_{12} μg./ml." should read "vitamin B_{12} mμg./ml.".

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